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# Study on the adaptive response to oxidative stress in *Saccharomyces cerevisiae*( Dissertation\_全文 )

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**Shingo Izawa**

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## General Introduction

The survival of living cells is dependent on their ability to sense alterations in the environment and to appropriately respond to the new situation. The yeast *Saccharomyces cerevisiae* has to adapt to a steadily changing environment in order to maintain a high proliferation rate. Alterations in the chemical or physical conditions of the cell that impose a negative effect on growth demand rapid stress responses which are essential for survival.

Stress response mechanisms aim to protect cells against detrimental effects of stresses and to repair damages. Therefore the stress responses lead to adjustment of metabolism and other cellular processes to the new status (Figure 1). The stress responses not only result in the repair of damage that has occurred but also lead to the acquisition of stress tolerance and thus to establishment of mechanisms that prevent damages which will occur subsequently. Indeed, exposure to a mild stress evokes improved resistance against severe stress. Thus, as a result of the stress response, the cells produce a number of proteins at a different level or with different activity than before stress exposure.

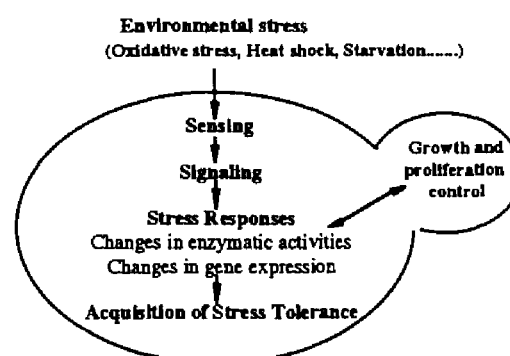


Fig.1 Stress Response in *Saccharomyces cerevisiae*

Oxidative stress is caused by reactive oxygen species (ROS) such as superoxide anion radical ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ) and hydroxyl radical ( $HO\bullet$ ). ROS are generated in respiration, and can attack almost all cell components including DNA, protein, and lipid membrane [1]. Additionally, ROS are known to be causative of degenerative diseases such as cancer and aging. Against ROS, the cells have some defensive mechanisms including superoxide dismutase, catalase, several peroxidases and antioxidants. Much work on the stress responses to oxidative stress has been carried out using bacterial cells, especially *Escherichia coli* and *Salmonella typhimurium* [2]. However, details of mechanisms of the stress response to oxidative stress in eukaryotic cells are currently unclear [3].

*Saccharomyces cerevisiae* is a truly an outstanding experimental model to dissect and understand the biochemical mechanisms by which cells sense and respond to oxidative stress. The ability to grow yeast cells both aerobically during which oxidative phosphorylation

(respiration) is the primary source of energy or anaerobically (with glycolysis as the sole source of ATP) makes *S. cerevisiae* an experimental system particularly suited for oxidative stress studies [4]. Since yeast strains which harbor deletions of important genes for protection against oxidative stresses are sensitive even to atmospheric oxygen concentrations, the ability to propagate such strains anaerobically or under extremely low oxygen tensions is advantageous. Additionally, *S. cerevisiae* is a eukaryotic microorganism with sixteen chromosomes and a mitochondrial genome, and the DNA of both has been completely sequenced [5]. Furthermore, given the mechanistic similarity with which yeast and humans are known to carry out a wide range of physiological processes, the ability to combine the power of molecular biology, classical genetic analysis, biochemistry, and cell biology all serve to make *S. cerevisiae* an ideal system to study oxidative stress.

In this thesis, the author investigated the mechanisms of adaptive response against  $H_2O_2$  in *S. cerevisiae*, using advantages of yeast described above. The author focused on the roles of catalases as scavenger enzymes for  $H_2O_2$  (Chapter I), glutathione as a low molecular intracellular thiol (Chapter II), and the recycling of glutathione (Chapter III) in the stress response to  $H_2O_2$ . The author also investigated the roles of other intracellular thiols, thioredoxin, glutaredoxin, and Yap1 (Chapter IV). Yap1 is a oxidative stress responsive transcription factor, and regulates the transcription of genes including the synthesis and recycling of glutathione or thioredoxin.

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## Chapter 1

### Importance of catalase in the adaptive response to hydrogen peroxide:

#### analysis of catalase-deficient *Saccharomyces cerevisiae*

##### 1-1. Introduction

All aerobic organisms use molecular oxygen ( $^3\text{O}_2$ ) for respiration or oxidation of nutrients to obtain the energy efficiently. During the reduction of molecular oxygen to water via the acceptance of four electrons, reactive oxygen species (ROS) such as superoxide radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\text{HO}^\bullet$ ) are generated. These species are capable of damaging DNA, protein and lipid membranes, and are known to be causative factors in degenerative diseases such as cancer. For defence against ROS, cells contain antioxidative enzymes such as superoxide dismutase, catalase, and several peroxidases as well as antioxidants such as ascorbate, tocopherol, and glutathione.

$\text{H}_2\text{O}_2$  is enzymatically catabolized in aerobic organisms by catalase and several peroxidases. In animals,  $\text{H}_2\text{O}_2$  is enzymatically detoxified by catalase and glutathione peroxidase (GPx). In these cells, and especially in human erythrocytes, the principal antioxidant enzyme for detoxification of  $\text{H}_2\text{O}_2$  has for a long time been considered to be GPx, as catalase has much lower affinity for  $\text{H}_2\text{O}_2$  than does GPx. Furthermore, it is reported that, in humans lacking erythrocyte catalase activity, these cells are not susceptible to haemolysis induced by oxidative stress [1]. Therefore it has been suggested and widely accepted that almost all  $\text{H}_2\text{O}_2$  is detoxified by GPx and that catalase has no role in the clearance of  $\text{H}_2\text{O}_2$ .

More recently, however, mammalian catalase and yeast catalase were found to contain tightly bound NADPH, and to require NADPH to prevent the formation of catalase compound II (inactive form) by  $\text{H}_2\text{O}_2$  [2-4]. This finding means that both GPx and catalase are dependent on NADPH, and raises the possibility that catalase, as well as GPx, is important in the detoxification of  $\text{H}_2\text{O}_2$ . Subsequently, several studies using acatalasaemic (catalase-deficient) cells and glucose-6-phosphate dehydrogenase (G6PDH)-deficient cells have reported that catalase plays an important role, in addition to that of GPx, in the protection against  $\text{H}_2\text{O}_2$  stress [5-9]. Controversy about the significance of catalase in the detoxification of  $\text{H}_2\text{O}_2$  in

animal cells continues to this day.

On the other hand, in bacterial organisms, it has been reported that catalase may not defend individual bacterial cells against  $H_2O_2$  stress. Ma and Eaton reported that individual catalase-deficient *Escherichia coli* cells showed an identical sensitivity to  $H_2O_2$  to that of wild-type cells, but high-density or colonial catalase-deficient cells showed greater susceptibility than wild-type cells under same conditions [10]. Similar results were reported in *Salmonella typhimurium* [11]. These findings suggested that catalase may function to protect groups of bacteria rather than discrete, isolated, cells.

In yeast *Saccharomyces cerevisiae*,  $H_2O_2$  is enzymatically catabolized by catalase and cytochrome c peroxidase. It is still controversial whether *S. cerevisiae* contains GPx or not. *S. cerevisiae* has two catalases, peroxisomal catalase (catalase A) and cytosolic catalase (catalase T), encoded by the *CTA1* and *CTT1* genes respectively [12, 13]. Much work has been done by Ruis and co-workers on the regulation of these genes and their transcription factors [14-19]. Despite the progress in elucidating the regulation of catalase genes, the real function of catalase in the oxidative stress response in *S. cerevisiae* is still in question.

In this chapter, the author investigated the role of catalase in the tolerance and the adaptive response to  $H_2O_2$  stress in *S. cerevisiae* using disruption mutants of the genes encoding the two catalases. The author has carefully tested the susceptibility of catalase-deficient mutants to  $H_2O_2$ , and discussed the differences of the role of catalase between yeast and other organisms.

## 1-2. Materials and Methods

### *Yeast strains and medium*

The yeast strains used in this study were as follows: YPH250 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*), YTT7 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ctt1::URA3*), YIT2 (*MATa his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 cta1::TRP1*), YWT1 (*MATa his3-Δ200 lys2-801 leu2-Δ1 ade2-101 cta1::TRP1 ctt1::URA3*). YPH250 was obtained from the Yeast Genetic Stock Center (University of California at Berkeley, CA, USA). Yeast cells were cultured in 50 ml of YPD medium (2% glucose, 2% peptone, 1% yeast extracts, pH 5.5) at 28 °C with reciprocal shaking (350 revs/min) in 200 ml Sakaguchi



flasks. Exponential- phase cells were harvested at an attenuation of the culture at 610 nm of 0.1 - 0.2. Stationary-phase cells were harvested after cultivation for 72 h.

#### *Disruption of the CTT1 and CTA1 genes and plasmid construction*

The *ctt1* deletion mutant was constructed by transforming YPH250 with the plasmid *ctt1::URA3* digested with *EcoRI/EcoRI*, and selecting for Ura<sup>+</sup> transformants, producing strain YTT7 (*ctt1Δ*). To delete the *CTA1* gene the following oligonucleotide primers were used in polymerase chain reaction (PCR) to amplify a 1.6 kb fragment containing the open reading frame of the *CTA1* coding sequence from genomic DNA: 5'-ATGTCGAAATTGGGACAAGA-3' and 5'-AAAATTTGGAGTTACTCGAAAGC-3'. PCR was carried out with the following condition: 94 °C for 1 min (denaturation), 55 °C for 2 min (annealing), 72 °C for 2 min (extension), 31 cycles. An *SspI/HincII* *CTA1* fragment was cloned into pUC19. The 0.8 kb *EcoRI/PstI* fragment from plasmid YRpG1, containing the *TRP1* gene, was inserted between the *HincII* site internal to the *CTA1* gene, constructing plasmid pCT721. Plasmid pCT721 was digested with *EcoRI/Eco47* to linearize the *cta1::TRP1* fragment, prior to the transformation of strain YPH250 to construct strain YTT2 (*cta1Δ*). A *ctt1* and *cta1* double deletion mutation was also constructed, producing strain YWT1 (*cta1Δ* & *ctt1Δ*). The replacement of the wild-type *CTT1* and *CTA1* alleles by the *ctt1::URA3* and *cta1::TRP1* disruption mutations was verified by PCR and catalase-activity staining [20]. Transformations were performed by the method of electroporation (Biorad; Gene pulser II).

#### *Enzymic Assays*

Cells were disrupted by vortexing with glass beads in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml pepstain A. Catalase activity was measured by the method of Roggenkamp *et al.* [21]. The rate of disappearance of H<sub>2</sub>O<sub>2</sub> was measured spectrophotometrically at 240 nm. One unit of catalase was defined as the amount of enzyme oxidizing 1 µmol H<sub>2</sub>O<sub>2</sub> per min at 25 °C. Cytochrome c peroxidase (CCP) activity was assayed by the method of Yonetani [22]. One unit of the activity was defined as the amount of enzyme oxidizing 1 µmol of cytochrome c per min at 25 °C. Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured according to

the method of Kornberg [23]. One unit of the activity was defined as the amount of enzyme oxidizing 1.0  $\mu\text{mol}$  of D-glucose 6-phosphate per min at 25 °C.

#### *Catalase-activity staining*

The method of catalase-activity staining was essentially the same as described by Clare *et al.* [20]. Cells in stationary phase were disrupted and cell-free extracts were applied to native electrophoresis, which was performed on 15% polyacrylamide gels. The gel was soaked in horseradish peroxidase (50  $\mu\text{g}/\text{ml}$ ) in 100 mM potassium phosphate buffer (pH 7.0) for 45 min.  $\text{H}_2\text{O}_2$  was then added to a concentration of 5.0 mM and soaking was continued for 10 min. The gel was then rapidly rinsed twice with distilled water and soaked into 0.5 mg/ml of diaminobenzidine in the potassium phosphate buffer until staining was complete.

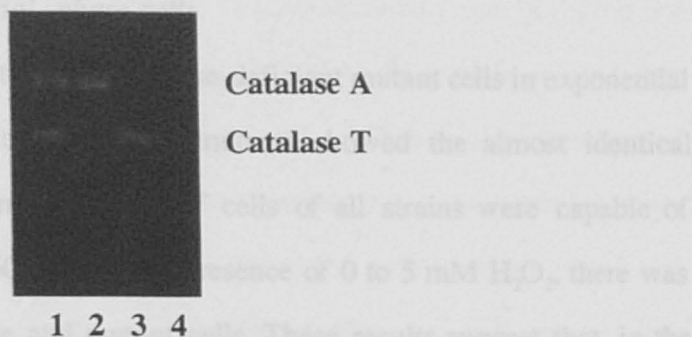
#### *H<sub>2</sub>O<sub>2</sub> treatment*

Conditions for the treatment of cells were essentially as described by Flattery-O'Brien *et al.* [24]. Cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial  $A_{610} = 0.1$ . This represented approximately  $2 \times 10^6$  cells per ml. To observe the susceptibility of yeast to  $\text{H}_2\text{O}_2$ , various concentrations of  $\text{H}_2\text{O}_2$  were added to 5 ml samples, and cell survival was monitored by taking samples at 20 min intervals, diluting in the same buffer and plating aliquots on YPD plates. For adaptation experiments, cells were pretreated with resuspending in fresh YPD medium containing a sublethal concentration of  $\text{H}_2\text{O}_2$  (0.2 mM) and incubated with shaking at 28 °C for 1 h. Pretreated cells were then harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4), and challenged with the lethal concentration of  $\text{H}_2\text{O}_2$  (2 mM).

### **1-3. Results**

#### *Construction of catalase-deficient mutants*

The catalase-deficient phenotypes of mutant strains were confirmed by catalase-activity staining. As showing in Figure 1, YTT7 (*ctt1* $\Delta$ ) expressed only catalase A (lane 2), YIT2 (*cta1* $\Delta$ ) expressed only catalase T (lane 3), and YWT1 (*cta1* $\Delta$  & *ctt1* $\Delta$ ) expressed neither catalase (lane 4).

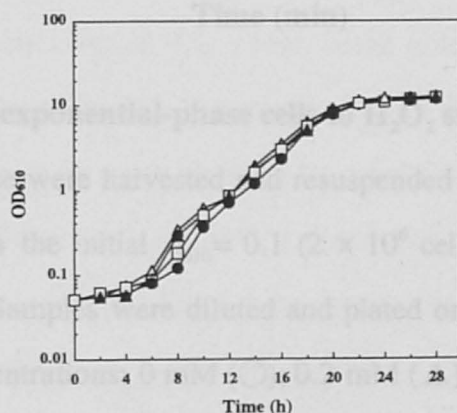


**Fig. 1 Catalase-activity staining**

Cell extracts of stationary phase cells of wild-type and catalase-deficient mutants were applied to native PAGE, and then the gel was stained. Upper band is catalase A activity, and lower band is catalase T. Lane 1, wild-type; 2, YTT7 (*ctt1Δ*); 3, YIT2 (*cta1Δ*); 4, YWT1 (*cta1Δ* & *ctt1Δ*).

#### *Effect of catalase on growth in batch culture*

The author investigated the effect of catalase deficiency on cell growth in batch culture without oxidative stress. Figure 2 shows the growth curves of wild-type and catalase-deficient mutant cells in YPD medium. All four strains showed similar growth rates. The presence or absence of catalase had little or no effect on growth rate under the condition without  $H_2O_2$  stress.

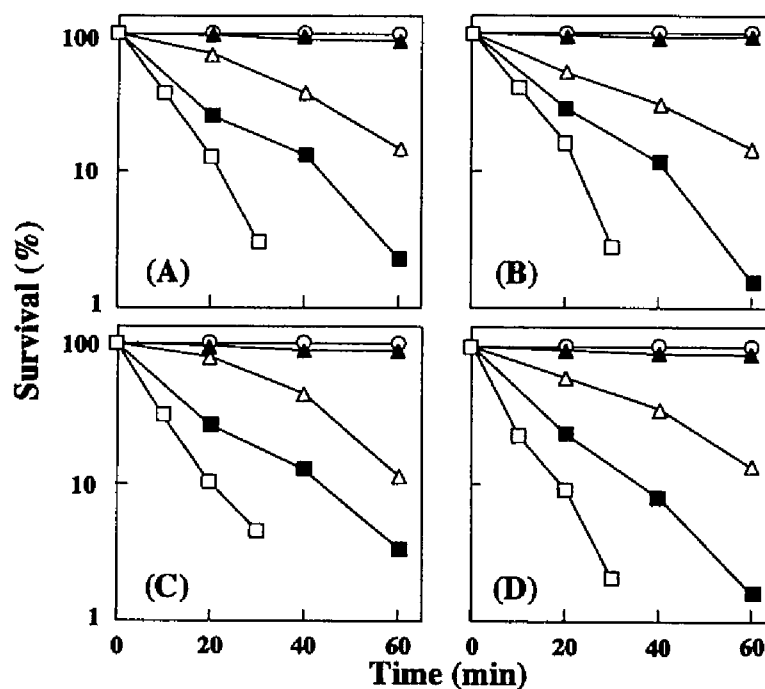


**Fig. 2 Comparison of growth with wild-type and catalase-deficient mutants**

Cells were cultured in YPD medium at 28 °C. Symbols are: YPH250 (●); YIT2 (Δ); YTT7 (▲); YWT1 (□).

### Susceptibility to $H_2O_2$ stress of exponential- phase cells

The susceptibility to  $H_2O_2$  stress of wild-type and catalase-deficient mutant cells in exponential phase was monitored (Figure 3). In log phase, all mutants showed the almost identical susceptibility to  $H_2O_2$  stress. Only a small number of cells of all strains were capable of surviving under 2 mM  $H_2O_2$  stress for 60 min. In the presence of 0 to 5 mM  $H_2O_2$ , there was no marked difference between wild-type and mutant cells. These results suggest that, in the exponential growth phase, catalase may not defend individual *S. cerevisiae* cells against  $H_2O_2$  stress, in analogy with bacterial cells [10, 11].



**Fig. 3** Susceptibility of exponential-phase cells to  $H_2O_2$  stress

Cells in exponential phase were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial  $A_{610} = 0.1$  ( $2 \times 10^6$  cells  $ml^{-1}$ ), and treated with various concentrations of  $H_2O_2$ . Samples were diluted and plated on YPD agar plates to monitor the cell-viability.  $H_2O_2$  concentrations: 0 mM (○); 0.2 mM (▲); 1 mM (Δ); 2 mM (■); 5 mM (□). (A), YPH250; (B), YIT2; (C), YTT7; (D), YWT1. Results represent the average of four independent experiments.

### Induction of adaptation to $H_2O_2$ stress

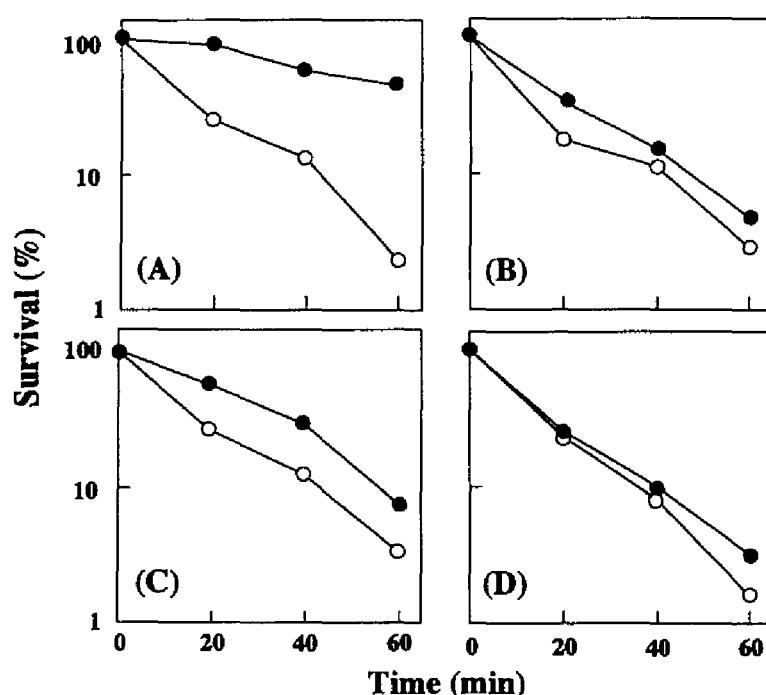
It is well known that cells pre-exposed to comparatively mild and sublethal stress conditions

show induction of resistance to subsequent lethal stress. The phenomenon was observed not only in bacterial cells but also in eukaryotic organisms, including *S. cerevisiae*, and was termed adaptation [25, 26]. The author investigated the role of catalase in the induction of adaptation to  $H_2O_2$  stress. Adaptation was induced by the pretreatment of exponential-phase cells with 0.2 mM  $H_2O_2$  for 60 min in YPD medium. The pretreated cells were harvested and then challenged to 2 mM  $H_2O_2$  stress in potassium phosphate buffer, and cell-survival was monitored.

With a 2 mM  $H_2O_2$  challenge, a difference of susceptibility was noted between wild-type cells and catalase-deficient mutants (Figure 4). Very little increase in tolerance was induced by the pretreatment in catalase-deficient mutant cells, especially in the catalase-deficient cells. The single-mutant catalase-deficient cells showed only slight increase of the tolerance against 2 mM  $H_2O_2$  stress (Figure 4 - B, C). There was almost no increase of the tolerance in acatalasemic cells (Figure 4 - D). These results suggest that catalase plays an important role on the induction of adaptation to  $H_2O_2$  stress.

#### *Susceptibility to $H_2O_2$ stress of stationary-phase cells*

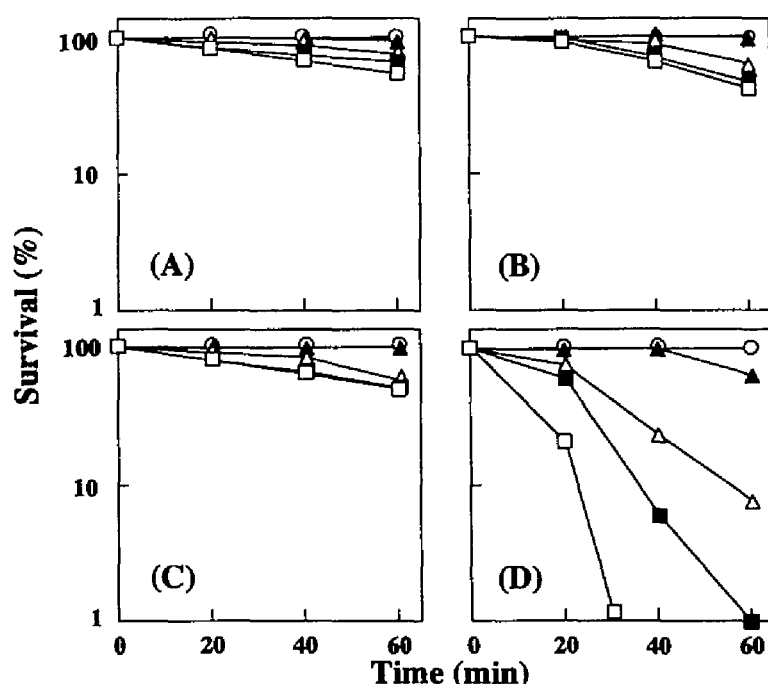
It has been reported that, if the microbial cells enter into the stationary growth phase, they acquire resistance to several environmental stresses such as heat, oxidative damage and osmotic stresses. It has been previously reported that *S. cerevisiae* in stationary phase acquires resistance to higher concentrations of  $H_2O_2$  stress, in the order of 10-20 mM, when compared with cells in exponential phase [26 -28]. The susceptibilities of wild-type and catalase-deficient mutant cells in stationary phase were thus investigated (Figure 5). The wild-type and the single-mutant catalase-deficient cells acquired a distinct resistance to  $H_2O_2$  stress on shifting from exponential to stationary phase. All of these three strains showed a similar susceptibility to  $H_2O_2$  stress. In the presence of 2 mM  $H_2O_2$ , almost all cells survived at 60 min, and approximately 50 % of total cells were alive under 20 mM  $H_2O_2$  stress for 60 min (Figures 5 - A, B, C). In contrast, the catalase-deficient cells displayed a greater susceptibility to high concentration (5 mM or more)  $H_2O_2$  stress. No viable cell was detected under 20 mM  $H_2O_2$  stress for 60 min, and approximately 90 % of the catalase-deficient cells were killed in only 5 mM  $H_2O_2$  by 60 min (Figure 5 - D).



**Fig. 4 Effect of catalase-deficiency on adaptation to 2 mM H<sub>2</sub>O<sub>2</sub>**

Cells in exponential phase were pretreated with 0.2 mM H<sub>2</sub>O<sub>2</sub> (●) for 1 h in fresh YPD medium, then resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial  $A_{610} = 0.1$ , and challenged to 2 mM H<sub>2</sub>O<sub>2</sub>. (○), cells of log phase were directly challenged to 2 mM H<sub>2</sub>O<sub>2</sub> without pretreatment. (A), YPH250; (B), YIT2; (C), YTT7; (D), YWT1. Results represent the means of four independent experiments.

Though the catalase-deficient cells were much more sensitive to H<sub>2</sub>O<sub>2</sub> stress than the wild-type and the single-mutant catalase-deficient cells of stationary phase, the catalase-deficient cells acquired the resistance to H<sub>2</sub>O<sub>2</sub> stress on the shift from exponential-phase to stationary-phase. More than 80 % of the catalase-deficient cells of stationary phase survived, whereas approximately 90 % of the catalase-deficient cells of exponential-phase were killed by 2 mM H<sub>2</sub>O<sub>2</sub> stress for 60 min (Figure 3 - D, 5 - D). Apparently the catalase-deficient mutant cells of stationary phase were much more resistant to H<sub>2</sub>O<sub>2</sub> stress than the cells of exponential-phase.



**Fig. 5 Susceptibility of stationary-phase cells to H<sub>2</sub>O<sub>2</sub> stress**

Cells in stationary phase were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial  $A_{610} = 0.1$ , and treated with various concentrations of H<sub>2</sub>O<sub>2</sub>. Samples were diluted and plated on YPD agar plates to monitor the cell viability. H<sub>2</sub>O<sub>2</sub> concentrations: 0 mM (○); 2 mM (▲); 5 mM (△); 10 mM (■); 20 mM (□). (A), YPH250; (B), YIT2; (C), YTT7; (D), YWT1. Results represent the means of four independent experiments.

#### *Change of enzyme activities*

The activities of catalase, cytochrome *c* peroxidase (CCP), and glucose-6-phosphate dehydrogenase (G6PDH) were measured under three different conditions as follows: exponential-phase cells without H<sub>2</sub>O<sub>2</sub> treatment, exponential-phase cells pretreated with 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min, and stationary-phase cells.

#### *(Catalase)*

Table 1 shows the change of catalase-activity in each strain under the various conditions, and relative activities are shown in Figure 6. In wild-type cells in exponential-phase, the activity

of catalase increased 1.7 fold by treatment with 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min (the pretreatment condition of the adaptation experiment). It has already been reported that expression of catalase is induced by many kinds of stresses, including oxidative stress, and by entering stationary phase [16, 29, 30]. Jamieson *et al.* reported that the transcription of the *CTT1* gene was induced 2-fold, and transcription of the *CTA1* gene was slightly induced, by H<sub>2</sub>O<sub>2</sub> [28]. Each catalase-deficient single mutant, *cta1Δ* and *ctt1Δ*, shows approximately half of catalase activity of the wild-type in exponential phase. On the pretreatment, the catalase activities of these two mutants increased approximately 2-fold; however, each activity was at the most equal to or less than that of non-pretreated wild-type cells (Figure 6).

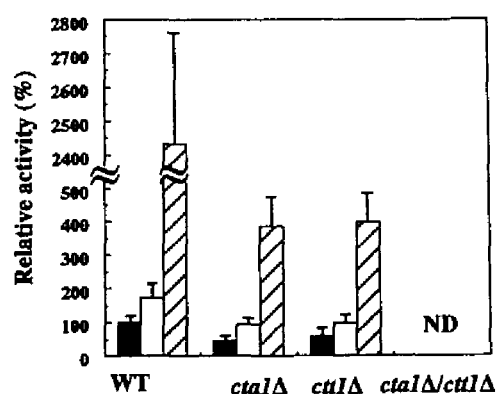
The activity of catalase increased dramatically (more than 20 fold) on transition from the exponential to the stationary phase in wild-type cells. The catalase-activities of *cta1Δ* cells and *ctt1Δ* cells of stationary phase were also increased 6 - 8-fold in comparison with those in exponential phase. No activity was detected in catalase-deficient cells under any conditions.

**Table 1 Effect of conditions on catalase activity\* in the various *S. cerevisiae* strains**  
Where applicable, H<sub>2</sub>O<sub>2</sub> was added at 0.2 mM for 60min. Values are means ± S. E. M. of four independent experiments. ND: Not detected.

Strain	Exponential-phase cells		Stationary-phase cells
	w/o H <sub>2</sub> O <sub>2</sub>	with H <sub>2</sub> O <sub>2</sub>	
YPH250 (wild-type)	1.32±0.28	2.28±0.59	32.21±4.20
YIT2 ( <i>cta1Δ</i> )	0.61±0.15	1.24±0.41	5.10±1.24
YTT7 ( <i>ctt1Δ</i> )	0.77±0.33	1.30±0.49	5.23±1.18
YWT1 ( <i>cta1Δ/ctt1Δ</i> )	ND	ND	ND

\*U/mg protein; 1 unit (U) of catalase was defined as the amount that catalyzes the degradation of 1 μmol H<sub>2</sub>O<sub>2</sub> per minute at 25 °C.





**Fig. 6 Change in the relative activities of catalase**

The catalase-activity of non-treated wild-type cells of exponential phase (1.32 u/mg of protein) was taken as 100 %. Cell conditions were as follows: filled bar, exponential phase cells without pretreatment; white bar, exponential phase cells treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> for 1 h in fresh YPD; hatched bar, stationary phase cells. ND: not detected.

(Cytochrome *c* peroxidase)

CCP also detoxifies H<sub>2</sub>O<sub>2</sub> in addition to catalase. The activities of CCP were shown in Table 2. There was no significant difference in CCP activity among the wild-type and mutants. The CCP activity of each strain increased 2.4 - 2.7-fold by pretreatment with 0.2 mM H<sub>2</sub>O<sub>2</sub> in exponential phase, and was increased 4 - 5-fold by the shift to the stationary phase.

**Table 2 Effect of conditions on CCP activity\* in the various *S. cerevisiae* strains**

Where applicable, H<sub>2</sub>O<sub>2</sub> was added at 0.2 mM for 60min. Values are means ± S. E. M. of four independent experiments.

Strain	Exponential-phase cells		Stationary-phase cells
	w/o H <sub>2</sub> O <sub>2</sub>	with H <sub>2</sub> O <sub>2</sub>	
YPH250 (wild-type)	4.1±0.6	11.2±0.9	18.3±3.4
YIT2 ( <i>ctalΔ</i> )	4.2±0.1	10.0±0.5	19.8±2.2
YTT7 ( <i>ctl1Δ</i> )	3.6±0.2	9.7±0.2	18.7±4.3
YWT1 ( <i>ctalΔ/ctl1Δ</i> )	3.5±0.1	9.3±0.7	14.7±2.2

\*mU/mg protein; 1 unit (U) of cytochrome *c* peroxidase was defined as the amount that oxidizes 1 μmol of cytochrome *c* per minute at 25 °C.

(Glucose-6-phosphate dehydrogenase)

Yeast catalases contain tightly bound NADPH. NADPH prevents compound II accumulation [2]. G6PDH is the key enzyme for the generation of NADPH via the pentose phosphate cycle. The presence of G6PDH and glucose 6-phosphate serves to keep the catalase-bound NADPH fully reduced and to keep catalase fully active. The activities of G6PDH under various conditions are shown in Table 3. All strains showed the similar patterns of G6PDH activity. The G6PDH activity of each strain was increased approximately 1.5-fold by pretreatment with 0.2 mM H<sub>2</sub>O<sub>2</sub> in the exponential-phase, and decreased 20-30% by the shift to the stationary phase.

**Table 3 Effect of conditions on G6PDH activity\* in the various *S. cerevisiae* strains**

Where applicable, H<sub>2</sub>O<sub>2</sub> was added at 0.2 mM for 60min. Values are means  $\pm$  S. E. M. of four independent experiments.

Strain	Exponential-phase cells		Stationary-phase cells
	w/o H <sub>2</sub> O <sub>2</sub>	with H <sub>2</sub> O <sub>2</sub>	
YPH250 (wild-type)	447.2 $\pm$ 7.2	685.6 $\pm$ 66.0	319.7 $\pm$ 12.7
YIT2 ( <i>cta1</i> $\Delta$ )	425.2 $\pm$ 11.2	792.8 $\pm$ 9.6	361.0 $\pm$ 15.3
YTT7 ( <i>cta1</i> $\Delta$ )	431.2 $\pm$ 50.8	842.4 $\pm$ 24.8	348.8 $\pm$ 21.1
YWT1 ( <i>cta1</i> $\Delta$ / <i>cta1</i> $\Delta$ )	544.8 $\pm$ 13.2	874.4 $\pm$ 50.0	338.2 $\pm$ 16.3

\*mU/mg protein; 1 unit (U) of glucose-6-phosphate dehydrogenase was defined as the amount that oxidizes 1  $\mu$ mol of D-glucose 6-phosphate per minute at 25  $^{\circ}$ C.

#### 1-4. Discussion

The focus of this study was to clarify the role of catalase in the response to oxidative stress in *S. cerevisiae*. To accomplish this objective, the author used and analyzed catalase-deficient mutants. In the absence of  $H_2O_2$  stress, the catalase-deficient yeast cells showed an identical growth rate to that of wild-type cells (Figure 2). This result indicates that catalase may not function in scavenging endogenous  $H_2O_2$  generated in metabolic reactions such as respiration or  $\beta$ -oxidation in growing yeast cells. It is likely that in *S. cerevisiae*  $H_2O_2$  is practically scavenged under non-oxidative stress condition by CCP or other mechanisms, but not by catalase. It has been suggested that in human erythrocytes all  $H_2O_2$  is practically scavenged by GPx, not by catalase, under physical conditions. However, some studies have indicated that catalase is equally important with GPx in detoxification of  $H_2O_2$  in human erythrocytes [5, 7-9]. In *S. cerevisiae*, the author did not obtain any results supporting the idea that catalase also plays a similarly important role to peroxidase under normal conditions. The results in this study may give a clue to solving the controversy about the role of catalase in erythrocytes.

In the exponential growth phase, there was no difference in susceptibility to  $H_2O_2$  stress between the wild-type and mutant yeast cells (Figure 3). Catalase-activities in *cta1* $\Delta$  cells and *ctl1* $\Delta$  cells were approximately half of that in wild-type, respectively, and in the catalase-deficient cells catalase-activity was not detected at all (Table 1, Figure 6). Despite distinct difference in catalase-activity, no difference was observed about the susceptibility to  $H_2O_2$  stress in log phase cells (Figure 3). These results indicate that catalase does not function as a scavenger for  $H_2O_2$  in actively growing cells. It is well known that the expression of catalase genes of *S. cerevisiae* is regulated by glucose and cAMP [15, 31]; thus catalase activity in exponential-phase is relatively lower than in stationary phase. Furthermore, there was no significant difference between wild-type and mutant cells in CCP-activity (Table 2). These facts further support the idea that catalase may not defend growing cells against  $H_2O_2$  stress. It seems that exponential-phase cells accommodate and respond to  $H_2O_2$  stress using mainly CCP or other systems without catalase.

The author investigated the role of catalase in the adaptive response to  $H_2O_2$  stress.

The acquisition of tolerance by mutant cells on pretreatment with 0.2 mM  $H_2O_2$  was imperfect in comparison with that by wild-type cells (Figure 4). With 2 mM  $H_2O_2$  challenge, relatively high concentration of  $H_2O_2$ , very little increase in tolerance was observed in mutant cells, especially in the catalase-deficient cells (Figure 4). Catalase activity was increased 1.5 - 2.0-fold by the pretreatment in wild-type and single-mutant catalase-deficient cells (Table 1, Figure 6). However, the catalase-activities of pretreated single mutants were the same as or lower than that of non-pretreated wild-type cells (*cta1* $\Delta$ : 93.9 %, *ctt1* $\Delta$ : 98.5 % of the activity of non-pretreated wild-type), and approximately half that of pretreated wild-type (Table 1, Figure 6). In catalase-deficient cells catalase-activity was not detected at all.

The *de-novo* synthesis of at least 21 proteins is increased on adaptation to  $H_2O_2$  in *S. cerevisiae* [32], and the expression of catalase is also inducible by oxidative stress [16, 18]. Experiments in this chapter confirmed the induction of not only catalase but also CCP-activity on adaptation of yeast cells (Table 2). There was no distinct difference of CCP-activity among all four strains under any conditions. In each strain, CCP-activity increased 2 - 3-fold by pretreatment with 0.2 mM  $H_2O_2$  (Table 2). However, a difference was observed in the cell survival on subsequent treatment with 2 mM  $H_2O_2$  between wild-type and catalase-deficient mutant cells (Figure 4). Without an increase of catalase activity, i.e. with only the increase of CCP and other defensive activities, it would be almost impossible for the cells to acquire the sufficient increase of tolerance in the adaptive response of *S. cerevisiae*. These results indicate that the induction of catalase is as important as that of CCP in the adaptation against  $H_2O_2$ . Especially when challenged with higher concentrations of  $H_2O_2$ , an appropriate increase in catalase activity seems to be necessary. From these results, the author concluded that not only the increase in CCP-activity but also appropriate levels of catalase-activity are essential to acquire the sufficient adaptation response.

On shifting from the exponential to the stationary phase, an increase in tolerance to  $H_2O_2$  stress was observed in all four strains. Even in the catalase-deficient yeast cells a remarkable increase in tolerance to  $H_2O_2$  was observed on the shift in growth phase (Fig. 3-D and 5-D). A significant increase in CCP-activity was observed in stationary phase catalase-deficient cells and wild-type cells (Table 2). Thus the increase in tolerance to  $H_2O_2$  in the catalase-deficient cells is dependent on the induction of CCP and other defense systems. A

difference in susceptibility between wild-type and the catalase-deficient cells is reflected by the absence of catalase in the latter, which indicates that catalase is essential in order to acquire maximal tolerance on shifting to stationary phase. On the other hand, both of single-mutant cells, *cta1Δ* and *ctt1Δ*, showed a similar susceptibility to  $H_2O_2$  to that of wild-type cells in the stationary phase. There were no distinct difference in CCP- and G6PDH-activity, and catalase-activities of *cta1Δ* cells and *ctt1Δ* cells of stationary phase increased 6 - 8 fold in comparison with those in the exponential phase. These results suggest that a catalase activity of approximately 5.00 units/mg of protein is enough in order to acquire maximal tolerance to  $H_2O_2$  on shifting to stationary phase. Furthermore, the localization of catalase, in peroxisome or in the cytosol, apparently has little or no effect on susceptibility to  $H_2O_2$ , and catalase A and catalase T seem to be able to compensate each other in the defense of yeast cells against  $H_2O_2$  stress.

G6PDH activity was also increased approximately 1.5-fold on pretreatment with 0.2 mM  $H_2O_2$ , but in contrast it was decreased in the stationary phase in all strains (Table 3). It has been previously reported that intracellular glutathione plays an important role in the adaptive response in *S. cerevisiae*, and also G6PDH is induced to recycle glutathione [33]. In addition, G6PDH is essential for the defense against oxidative stress in mouse [34]. As shown in Table 3, there was no distinct difference of the activity between wild-type and mutant cells, and an increase of activity on  $H_2O_2$  pretreatment was observed even in catalase-deficient mutant cells. In the stationary phase, a decrease in G6PDH activity was observed, in contrast with the increase in catalase activity (Table 1, 3). An effect of catalase-deficiency on the change in G6PDH activity was not observed in this study.

In *E. coli*, expression of the catalase genes (*katE* and *katG*) is induced in the stationary phase and by  $H_2O_2$  stress under the control RpoS and OxyR regulon [35, 36]. On the other hand, it has been reported that the protective effect of bacterial catalase is cell-density-dependent. Thus, under low cell-density condition catalase deficiency has no effect on the susceptibility to  $H_2O_2$  in bacterial cells [10, 11, 37]. It is thought that catalase is able to decrease  $H_2O_2$  by a mass effect at high cell-density. At low cell-density, catalase is incapable of maintaining an internal/external concentration gradient of  $H_2O_2$ , since the rate of  $H_2O_2$  permeation into each individual cell appears to exceed the rate of degradation by catalase. In

this study, all experiments on susceptibility and adaptation were carried out at low cell-density ( $2 \times 10^6$  cells ml<sup>-1</sup>). Surprisingly, in yeast cells of stationary phase, the effect of catalase to H<sub>2</sub>O<sub>2</sub> stress was observed even under conditions of low cell-density. The catalase -deficient yeast cells in stationary phase were distinctly more sensitive against H<sub>2</sub>O<sub>2</sub> stress than wild-type and single-mutant cells at low cell-density (Figure 5). Moreover, catalase played an important role in the acquisition of tolerance in the adaptation of exponential-phase cells (Figure 4). Thus in *S. cerevisiae*, catalase could protect cells even at low cell-density in the adaptive response and in stationary-phase cells. It seems that there is difference in the role of catalase between yeast and bacterial cells. A difference in the role of glutathione in the response to oxidative stress has also been observed between *E. coli* and *S. cerevisiae* [33, 38]. The defences mechanism of yeast seems more complicated than that of bacterial cells [39]. The difference in the stress response systems may have some interesting relationship with the evolution of organisms as mentioned by Ma and Eaton [10].

In conclusion, in *S. cerevisiae*, catalase does not play an important role to detoxification of H<sub>2</sub>O<sub>2</sub> under physiological conditions. However, under condition of stress, in the stationary phase or in the adaptive response, catalase is necessary and plays an important role in the acquisition of a sufficient increase of tolerance to H<sub>2</sub>O<sub>2</sub> stress. Thus catalase seems to be necessary in an 'emergency'. Further studies on the role of catalase in the response to other kinds of stress are underway to clarify the function of catalase in *S. cerevisiae*.

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## Chapter 2

### Effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*

#### 2-1. Introduction

All aerobic organisms use molecular oxygen ( $^3\text{O}_2$ ) for respiration or oxidation of nutrients to obtain the energy efficiently. During the reduction of molecular oxygen to water through acceptance of four electrons, active oxygen species such as superoxide anion radical ( $\text{O}_2^{\cdot-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\text{HO}^\bullet$ ) are generated. These active oxygen species attack almost all cell components, DNA, protein and lipid membrane. Active oxygen species are known to be causative of degenerative diseases such as cancer [1, 2]. Against such active oxygens, the cells have some defensive mechanisms including superoxide dismutase, catalase, several peroxidases and antioxidants such as ascorbate, tocopherol, uric acid,  $\beta$ -carotene and glutathione.

Glutathione is widely found in microorganisms, plants and animals. It has various functions in the defense against oxidative stress and xenobiotic toxicity [3-6]. In animals,  $\text{H}_2\text{O}_2$  generated through respiration is scavenged in mitochondria by glutathione peroxidase (GPx). Thus glutathione has an essential function in mitochondria under normal physiological condition in animals, and glutathione deficiency leads to several diseases by mitochondria damage [7, 8].

Much of previous work about oxidative stress responses of *S. cerevisiae* has focused on the stress-inducible proteins and membrane lipid unsaturation, and has shown that there are distinct inducible stress responses to both peroxides and superoxide [9-12]. Davies *et al.* have reported that adaptation to  $\text{H}_2\text{O}_2$  requires protein synthesis and expression of at least 21 proteins increased following  $\text{H}_2\text{O}_2$  adaptation [13]. On the other hand, Wu and Moye-Rowley reported that expression of *GSH1* gene encoding  $\gamma$ -glutamylcysteine synthetase is regulated by yAP-1, a transcriptional regulator of the oxidative stress response in *S. cerevisiae* [14].  $\gamma$ -Glutamylcysteine synthetase is a key enzyme for glutathione biosynthesis. However, informations about the role of glutathione on the stress response in *S. cerevisiae* are largely lacking. In this chapter, the author examined the function of intracellular glutathione on

H<sub>2</sub>O<sub>2</sub> stress response in yeast.

## 2-2. Materials and Methods

### *Yeast strains and media S. cerevisiae*

S288C (*MATa SUC2 mal mel gal2 CUP1*) was obtained from Yeast Genetic Stock Center, University of California, Berkeley, USA. YNN27 (*MATα trp1 ura3*) and YH-1 (*MATα trp1 ura3 gsh1*) were kindly gifted from Dr. Y. Ohtake, Asahi Breweries Ltd. Cells of S288C were cultured in YPD medium (2% glucose, 2% peptone, 1% yeast extracts) at 28 °C with reciprocal shaking. YNN27 and YH-1 were cultured in SD medium (2% glucose, 0.67% yeast nitrogen base, 20μg/ml of L-tryptophan and uracil). Exponential-phase cells were harvested at the optical density of the culture at 610 nm (OD<sub>610</sub>) of 0.1. Stationary phase cells were harvested after the cultivation for 72 h.

### *H<sub>2</sub>O<sub>2</sub> treatment*

Condition for the treatment of the cells was essentially same as that described by Flattery-O'brien *et al.* [11]. Cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial OD<sub>610</sub> = 0.1. To observe the sensitivity of yeast to H<sub>2</sub>O<sub>2</sub>, various concentrations of H<sub>2</sub>O<sub>2</sub> were added to 5 ml samples, and cell survival was monitored by taking samples at 15 min intervals, diluting by the same buffer and plating aliquots on YPD plates. For adaptation experiments, cells were resuspended in fresh YPD medium containing a sublethal concentration of H<sub>2</sub>O<sub>2</sub> (0.2 mM) and incubated with shaking at 28 °C for 60 min. Pretreated cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4), and challenged to the lethal concentration of H<sub>2</sub>O<sub>2</sub> (2 mM). SD medium was used instead of YPD for YNN27 and YH-1, and stress conditions were as follows: first stress; 0.01 mM for 60 min, second stress; 0.1 mM.

### *Assay of glutathione producing activity*

Assay of glutathione-producing activity and extraction of glutathione from cells were carried out essentially same as that described by Murata *et al.* [15]. Cells were incubated in a mixture containing 0.5 M glucose, 0.01 M MgCl<sub>2</sub>, 0.02 M L-glutamate, 0.02 M L-cysteine, 0.02 M

glycine and 0.1 M potassium phosphate buffer (pH 7.4) at 28 °C for 60 min with shaking. After the incubation, cells were collected by centrifugation and washed twice with distilled water. Glutathione in the cells was then extracted and determined by the method of Tietze [16]. Glutathione producing activity was expressed as the amount of glutathione formed in 60 min per g (as wet weight) of the cells.

#### *Enzyme activities*

Cells were disrupted by vortex with glass beads in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 1 µg/ml pepstatin A. Glutathione reductase activity was measured according to the method of Racker [17]. Glucose-6-phosphate dehydrogenase activity was measured by the method of Kornberg and Horecker [18]. Protein was measured by the method of Lowry *et al.* [19].

#### *Depletion of intracellular glutathione*

To decrease intracellular glutathione content, cells were incubated in YPD medium containing 0.5 mM 1-chloro-2,4-dinitrobenzene (CDNB) for 60 min. To increase glutathione content, cells were incubated in the mixture containing L-glutamate, L-cysteine and glycine as described above for 60 min. To inhibit GSH-I activity, cells were incubated in YPD medium containing 1 mM L-buthionine sulfoximine (BSO).

### **2-3. Results**

#### *Effect of glutathione content on the sensitivity to H<sub>2</sub>O<sub>2</sub>*

It has been reported that if the cells of microorganisms enter into the stationary phase, the cells acquire the resistance against several environmental stresses such as heat, oxidative damage and osmotic stress [20-22]. The author also confirmed that cells of *S. cerevisiae* S288C in stationary phase showed higher resistance against H<sub>2</sub>O<sub>2</sub> (data not shown), which was previously reported by Steels *et al.* [23]. It was thought to be due to the increased expression of some genes encoding stress shock proteins. In the meanwhile, as shown in Table 1, intracellular glutathione content of cells in stationary phase was approximately 3-fold higher than that in log phase cells. Therefore, the author speculated that intracellular

glutathione level may also be one of the factors that determine the resistance against several stresses.

Intracellular glutathione (GSH+GSSG) can be depleted by the treatment with CDNB. Approximately 50% of intracellular glutathione was abolished by treatment of the cells with 0.5 mM CDNB for 60 min (Table 1). Figure 1 shows the effect of CDNB on the sensitivity of the cells against  $H_2O_2$ . The cells pretreated by CDNB became hypersensitive to  $H_2O_2$ .

**Table 1 Intracellular glutathione content under several conditions**

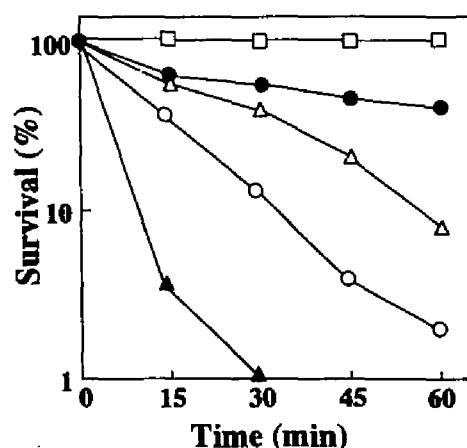
	Glutathione content ( $\mu\text{mol/g cell}$ )
Exponential-phase cell	$2.22 \pm 0.04$
Stationary phase cell	$6.89 \pm 0.07$
CDNB treatment <sup>a</sup>	$1.23 \pm 0.08$
BSO treatment <sup>b</sup>	$1.98 \pm 0.07$
Incubation with amino acids <sup>c</sup>	$18.47 \pm 0.88$
0.2 mM $H_2O_2$	$2.47 \pm 0.03$

Values are shown as the average of four independent experiments.

<sup>a</sup> Cells were treated with 0.5 mM CDNB for 60 min.

<sup>b</sup> Cells were treated with 1 mM BSO for 60 min.

<sup>c</sup> Cells were incubated with 0.02 M L-glutamate, L-cysteine and glycine for 60 min.



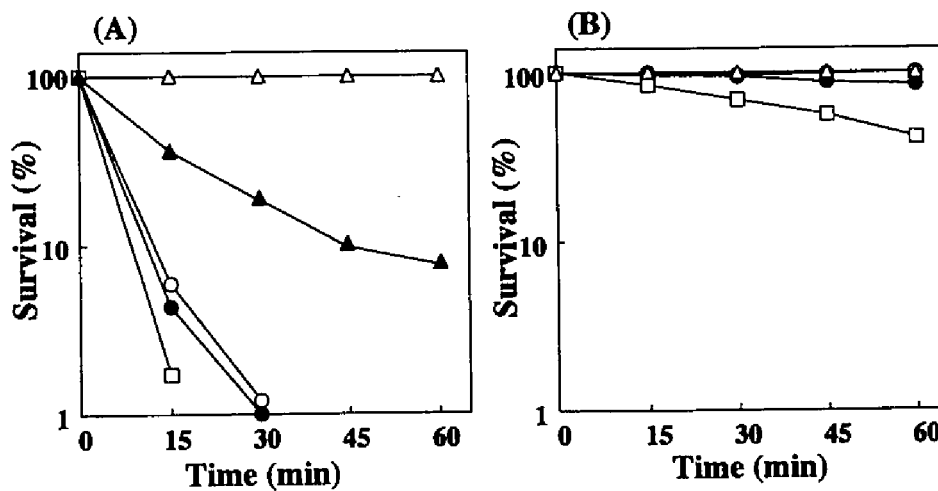
**Fig. 1 Effect of intracellular glutathione content on sensitivity to  $H_2O_2$**

Cells of S288C in log phase were treated with 0.5 mM CDNB (▲), 1 mM BSO (○) for 60 min in YPD medium at 28 °C, or incubated in a mixture containing 0.02 M L-glutamate, L-cysteine and glycine for 60 min at 28 °C (●), respectively, and then challenged to  $H_2O_2$  (2 mM). □, cells were directly challenged to  $H_2O_2$  (2mM) without pretreatment. Δ, cells were not treated with any chemicals (as control).

Biosynthesis of glutathione is catalyzed by two enzymes, i. e., GSH-I ( $\gamma$ -glutamylcysteine synthetase) and GSH-II (glutathione synthetase). BSO is a potent inhibitor of GSH-I, and the glutathione content of the cells treated by BSO (1 mM for 60 min) was also decreased compared with that of the control cells (Table 1). Pretreatment of the cells by BSO also made the cells sensitive to  $H_2O_2$  (Fig. 1).

Glutathione is a tripeptide consisting of L-glutamate, L-cysteine and glycine. Incubation of the cells with these amino acids increased the intracellular glutathione level (Table 1) [15], and consequently the cells showed the higher resistance against  $H_2O_2$  (Fig. 1).

To confirm the effect of glutathione content on the stress response, the sensitivity of glutathione biosynthesis-deficient mutant YH-1 to  $H_2O_2$  was investigated. YH-1 is lacking the GSH-I activity and is not containing glutathione in the cells [24]. As shown in Fig. 2, YH-1 was much more sensitive to  $H_2O_2$  than YNN27, which is a parent strain of YH-1. YNN27 could survive in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM  $H_2O_2$  for 60 min (Fig. 2A), whereas 99% of the mutant cells were killed under the same conditions (Fig. 2B).



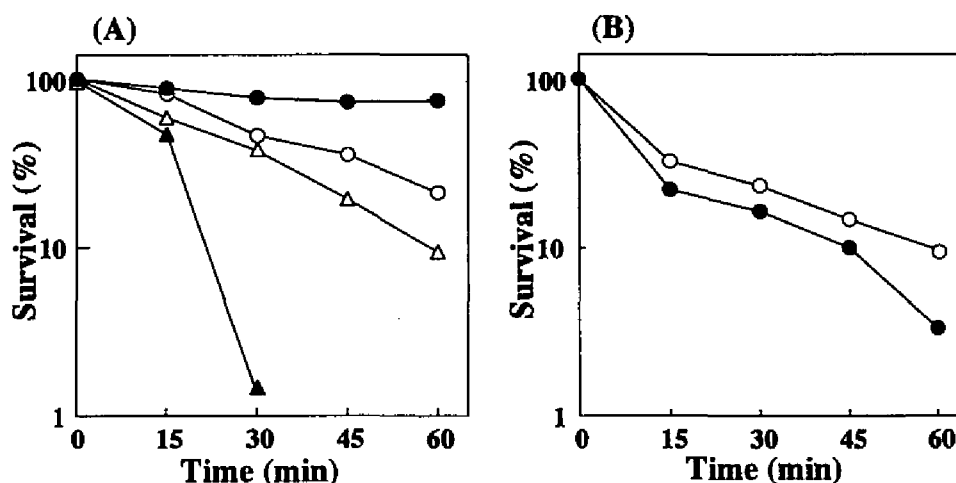
**Fig. 2 Sensitivity of glutathione-deficient mutant YH-1 (A) and its parental strain YNN27 (B) to  $H_2O_2$**

Cells were cultured in SD medium to exponential-phase and treated with various concentrations of  $H_2O_2$  for 60 min. Samples were diluted and plated on YPD agar plates to monitor the cell viability.  $H_2O_2$  concentrations and symbols were: 0 mM ( $\Delta$ ); 0.1 mM ( $\blacktriangle$ ); 0.2 mM ( $\bigcirc$ ); 0.5 mM ( $\bullet$ ); 1 mM ( $\square$ ).

### *Effect of glutathione content on the adaptation to H<sub>2</sub>O<sub>2</sub>*

It has been known that pretreatment of the cells by sublethal concentration of H<sub>2</sub>O<sub>2</sub> renders the cells resistance against subsequent treatment by lethal concentration of H<sub>2</sub>O<sub>2</sub>. The phenomenon was observed in both of prokaryotic and eukaryotic microorganisms, including *S. cerevisiae* [9, 11-13], and termed as adaptation.

The author also confirmed that adaptation was induced by pretreatment of *S. cerevisiae* S288C by sublethal concentration (0.2 mM) of H<sub>2</sub>O<sub>2</sub> (Fig. 3A). The adaptation was suppressed if the cells were treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> in the presence of 0.5 mM CDNB (Fig. 3A). Partial inhibition was also observed if the pretreatment by H<sub>2</sub>O<sub>2</sub> was carried out with 1 mM BSO (Fig. 3A). Furthermore, adaptation was not observed in the case of glutathione biosynthesis-deficient mutant YH-1 (Fig. 3B). These results suggest that intracellular content of glutathione affects the adaptation to oxidative stress caused by H<sub>2</sub>O<sub>2</sub>.



**Fig. 3 Effect of intracellular glutathione content on adaptation**

(A) Cells of S288C in exponential-phase were pretreated with 0.2 mM H<sub>2</sub>O<sub>2</sub> (●); 0.2 mM H<sub>2</sub>O<sub>2</sub> and 1 mM BSO (○); 0.2 mM H<sub>2</sub>O<sub>2</sub> and 0.5 mM CDNB (▲) for 60 min, respectively, and then challenged to H<sub>2</sub>O<sub>2</sub> (2mM). Δ, cells were directly challenged to H<sub>2</sub>O<sub>2</sub> (2mM) without pretreatment. (B) Cells of YH-1 in log phase were pretreated with 0.01 mM H<sub>2</sub>O<sub>2</sub> (●) for 60 min, and then challenged to H<sub>2</sub>O<sub>2</sub> (0.1 mM). ○, cells were directly challenged to H<sub>2</sub>O<sub>2</sub> (0.1 mM) without pretreatment.

### *Change of glutathione producing and recycling activities*

Activities of enzymes involved in biosynthesis and recycling of glutathione were measured. Glutathione producing activity slightly (approximately 10%) increased by treatment of the cells with 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Glutathione disulfide (GSSG) is reduced to a reduced form of glutathione (GSH) by glutathione reductase (GR) in the presence of NADPH. NADP<sup>+</sup> thus formed is reduced to NADPH by an action of glucose-6-phosphate dehydrogenase (G6PDH). Both GR and G6PDH are involved in the glutathione recycling system. As shown in Table 2, both enzyme activities increased approximately 1.5-folds after the treatment with 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Total intracellular glutathione content slightly increased by the same treatment (Table 1). These results suggest that *S. cerevisiae* cells seem to adapt to H<sub>2</sub>O<sub>2</sub> stress by increasing both glutathione content and glutathione recycling activities.

**Table 2** Change in enzyme activities with H<sub>2</sub>O<sub>2</sub> treatment

Treatment with H <sub>2</sub> O <sub>2</sub>	GSH-I + GSH-II <sup>a</sup>	GR <sup>b</sup>	G6PD <sup>c</sup>
Non treated	16.25±0.88 (100)	28.7±0.9 (100)	461±54 (100)
0.2 mM, 1h	17.81±2.03 (110±12)	41.7±4.3 (145±15)	607±131 (151±28)

Values are shown as the means of four independent experiments.

<sup>a</sup> μmol glutathione formed/h/g(wet-wt.)-cells

<sup>b</sup> mU/mg protein; 1 unit (U) of the activity was defined as the amount of enzyme reducing 1.0

<sup>c</sup> μmol of GSSG per min at 25 °C.

mU/mg protein; 1 unit (U) of the activity was defined as the amount of enzyme oxidizing 1.0 μmol of D-glucose 6-phosphate per min at 25 °C.

Parentheses show the relative activity of each enzyme. The activities of non-treated cells are relatively taken as 100 %.

## 2-4. Discussion

In mammalian system, Meister and Anderson proposed that glutathione protects the cells from oxidative damage [5]. Contrasting to this, Greenberg and Demple reported that *gshA::Tn10km* mutant of *Escherichia coli* K-12 has normal resistance against H<sub>2</sub>O<sub>2</sub> and cumene hydroperoxide [25]. The *gshA* gene encodes γ-glutamylcysteine synthetase (GSH-I). GSH-I is a rate-limiting enzyme in the biosynthesis of glutathione, and intracellular glutathione content in the *gshA::Tn10km* mutant decreased to <0.4% compared with that of wild type strain. Therefore, Greenberg and Demple concluded that intracellular glutathione did not protect *E. coli* from the oxidative damage.

On the other hand, the author demonstrated that intracellular glutathione played an

important role in the stress response to  $H_2O_2$  in *S. cerevisiae* using glutathione depleting agents and glutathione-deficient mutant. Sensitivity to  $H_2O_2$  increased if *S. cerevisiae* cells were treated by glutathione depleting agents such as CDNB and BSO, whereas incubation of the cells with amino acids (L-Glu, L-Cys, Gly) which constitute glutathione increased the intracellular glutathione content and subsequently the cells acquired the resistance against  $H_2O_2$ . *S. cerevisiae* YH-1, which is a glutathione-deficient mutant, was hypersensitive to  $H_2O_2$  compared with its parental strain, and did not show the adaptation. Cellular glutathione was not detected in the mutant deficient in *gsh1* gene in *S. cerevisiae* [24]. Therefore, *S. cerevisiae* may have a similar mechanism as observed in mammalian cells to protect the cells against the oxidative damages.

Many studies have been reported on the relationship between glutathione and stress response in animal cells [7, 8, 26-29]. In mammalian cells, glutathione peroxidase (GPx) has a primary function to scavenge  $H_2O_2$ .  $H_2O_2$  is reduced to  $H_2O$  by GPx. Glutathione in reduced form (GSH) is an electron donor for GPx reaction, and oxidized form of glutathione (GSSG) is reduced to GSH by glutathione reductase (GR) in the presence of NADPH.  $NADP^+$  is reduced to NADPH by glucose-6-phosphate dehydrogenase (G6PDH). In *E. coli* and *Salmonella typhimurium*, *gorA* gene, which encodes GR, is involved in the  $H_2O_2$ -inducible oxyR regulon [30]. As shown in Table 2, treatment of *S. cerevisiae* cells with sublethal concentration of  $H_2O_2$  induced both GR and G6PDH. Glutathione synthesizing activity was also slightly increased by  $H_2O_2$  treatment, and BSO which is a potent inhibitor of GSH-I suppressed the adaptation. Therefore, *de novo* synthesis of glutathione and recycling of glutathione are likely to be involved in the adaptation system in *S. cerevisiae*.

It has been previously reported that a yeast *Hansenula mrakii* has a membrane-bound GPx which was induced by lipid hydroperoxide [31-32]. In such a case, G6PDH was also induced to supply NADPH [33]. However, it is still controversial whether *S. cerevisiae* has GPx or not. If *S. cerevisiae* has a GPx, it is entirely reasonable that *S. cerevisiae* increases intracellular glutathione content as an electron donor for GPx reaction and adapts to  $H_2O_2$  stress in analogy with mammalian cells. In the case of *S. cerevisiae* does not have a GPx, the author is still able to give some explanations for the increase of intracellular glutathione content to protect the cell against oxidative stress. For example, glutathione can directly



scavenge the reactive oxygen species. It is well known that glutathione is a scavenger of HO• and singlet oxygen (<sup>1</sup>O<sub>2</sub>) [2, 3, 34]. HO• and O<sub>2</sub>•<sup>-</sup> are formed by Fenton reaction from H<sub>2</sub>O<sub>2</sub> in the presence of Fe(II). Alternatively, glutathione can reactivate disulfide bonds (-SS-) of some proteins to generate sulfhydryl (-SH) groups, which may be involved in essential sites of such proteins [3, 35]. It is likely that glutathione contributes to sustentation the functions of such proteins. Therefore, the increase of intracellular glutathione may be one of the adaptations to H<sub>2</sub>O<sub>2</sub> stress in *S. cerevisiae*.

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## Chapter 3

### Importance of glutathione recycling in the adaptive response to hydrogen peroxide in *Saccharomyces cerevisiae*

#### 3-1. Introduction

Cells pre-exposed to comparatively mild and sublethal stress situations activate the adaptive responses and acquire tolerance to subsequent more lethal stress. Such responses, named adaptation, are observed not only in bacterial cells but also in eukaryotic organisms, including *Saccharomyces cerevisiae* [1]. Detailed mechanisms of adaptation to oxidative stress have been characterized most extensively in bacterial cells [2], but have also been studied in yeast. In *S. cerevisiae*, at least 21 proteins are increased in response to exposure to  $H_2O_2$ , and a number of genes are transcriptionally regulated by  $H_2O_2$  stress [3-5]. Some investigators, including us, have shown that glutathione is essential as an antioxidant for adaptation to  $H_2O_2$  in *S. cerevisiae* [6-9]. Furthermore, glutathione-synthesis and glutathione-recycling activities are increased, and expression of the *GSH1* (encoding  $\gamma$ -glutamylcysteine synthetase) and the *GLR1* (glutathione reductase) genes is elevated under the control of yAP-1, a transcription activator in *S. cerevisiae* [10-12].

Glutathione ( $\gamma$ -glutamylcysteinylglycine) is a low-molecular-mass thiol that is widely distributed in micro-organisms, plants, and animals. It has various functions in the defense against oxidative stress and xenobiotic toxicity [13]. It can act as the electron donor for glutathione peroxidase (GPx) in animal cells, and also directly reacts with reactive oxygen species (ROS). The reduced form glutathione (GSH) is oxidized to glutathione disulfide (GSSG) by the GPx reaction as well as the reaction with ROS. GSSG is reduced and recycled by an NADPH-dependent reaction catalyzed by glutathione reductase (GR). GR is necessary for the maintenance of GSH levels in cells. NADPH, the reducing agent, is mainly generated by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) via the pentose phosphate pathway. G6PDH catalyzes the rate-limiting NADPH-producing step in the pentose phosphate pathway [14].

The author has reported that G6PDH and GR were induced simultaneously by oxidative stress in *S. cerevisiae* [6], whereas in another yeast, *Hansenula mrakii*, only G6PDH was

induced [15]. It has been also reported that G6PDH-deficient *S. cerevisiae* showed the hypersensitivity to oxidative stress [16-18]. These data strongly indicate that G6PDH plays an important role in the acquisition of tolerance against oxidative stress. However, the intrinsic function of G6PDH in the oxidative stress response in yeast is still obscure.

In humans, G6PDH deficiency is a common genetic disorder that can result in haemolytic anemia and enhanced oxidant sensitivity of erythrocytes [19]. In the absence of this enzyme, erythrocytes are unable to reduce GSSG using GR because of the lack of NADPH, and thus have low concentrations of GSH. Historically, the decrease in the cellular GSH was thought to account for the increased oxidant-sensitivity of G6PDH-deficient erythrocytes. However, Scott *et al.* have cast substantial doubts on the pre-eminent importance of GSH in the defense against H<sub>2</sub>O<sub>2</sub> stress. They demonstrated that GSH level did not affect the susceptibility to H<sub>2</sub>O<sub>2</sub> stress, using G6PDH-deficient human erythrocytes and acatalasaemic mice. No statistical correlation was found between GSH level and oxidant sensitivity of erythrocytes, but a strong correlation between NADPH level and oxidant sensitivity was observed [20-22].

In *Escherichia coli*, G6PDH is induced by the *soxRS* regulon in oxidative stress response [23]. However, it has been reported that glutathione and glutathione-recycling are not necessary for protection of *E. coli* cells against oxidative stress, because the *gshA::Tn10* mutant showed normal resistance to H<sub>2</sub>O<sub>2</sub> and cumene hydroperoxide [24]. The *gshA* gene encodes  $\gamma$ -glutamylcysteine synthetase (GSH-I), which catalyzes the rate-limiting step in GSH biosynthesis, and such a mutant has less than 0.4 % of wild-type GSH level. Furthermore, GR-deficient mutant (*gor*<sup>-</sup>) can keep intracellular glutathione highly reduced, suggesting that GR is not required for maintenance of GSH in *E. coli* [25]. The roles of glutathione and glutathione-recycling in the resistance to oxidative stress may be different among yeasts, animals, and bacterial cells.

The author is interested in the roles of G6PDH and glutathione recycling in the adaptive response to oxidative stress in *S. cerevisiae*. In this chapter, to estimate the importance of glutathione-recycling in the stress response, the author investigated whether or not the enhanced sensitivity of G6PDH-deficient yeast is caused by insufficient recycling of glutathione by analyzing phenotypes of the GR- and G6PDH-deficient mutants.

### 3-2. Materials and Methods

#### *Yeast strains and medium*

Strains of *S. cerevisiae* used in this study were as follows: YPH250 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*), GRK1 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 glr1Δ :: HIS3*), ZWK7 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 zwf1Δ :: URA3*), ZGK2 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52 zwf1Δ :: URA3 gnd1Δ::HIS3*). *S. cerevisiae* YPH250 was obtained from the Yeast Genetic Stock Center, University of California at Berkeley. Cells of *S. cerevisiae* were cultured in 50 ml YPD medium (1% yeast extract, 2% pepton, 2% glucose, pH 5.5) or SD minimal medium (2% glucose, 0.67% yeast nitrogen base w/o amino acids, pH 5.5) with appropriate amino acids and bases at 28 °C with reciprocal shaking (200 rev./min) in 300 ml Erlenmeyer flasks. Exponentially growing cells were harvested at  $A_{610}=0.2-0.3$ .

#### *Construction of disruption mutants and plasmids*

Disruption of the *ZWF1* gene, which encodes G6PDH, was done using pBM2720 that was kindly provided by Dr. M. Johnston [16]. pBM2720 was digested with *EcoRI* and *BamHI*, and the resulting fragment containing *zwf1::URA3* was introduced to *S. cerevisiae* YPH250 to yield a strain ZWK7 (*zwf1Δ*). The *GND1* gene, which encodes 6PGDH, was disrupted by the use of pBSK-*gnd1::HIS3*, that was kindly donated by Drs. H. Juhnke and K.-D. Entian [17]. The plasmid was digested with *ClaI* and *SacII*, and the resultant fragment carrying the *gnd1::HIS3* was introduced to the strain ZWK7 to produce a strain ZGK2 (*zwf1Δ/gnd1Δ*). Since a fission yeast *Schizosaccharomyces pombe* was reported to have a pathway that can skip the G6PDH reaction, and NADPH can be generated by 6PGDH reaction, we then constructed *zwf1Δ/gnd1Δ* double mutant to completely disrupt generation of NADPH in pentose phosphate pathway. To disrupt the *GLR1* gene which encodes GR, the following oligonucleotide primers were used in polymerase chain reaction (PCR) to amplify a 1.95 kb fragment containing the open reading frame of the *GLR1* gene from genomic DNA: 5'-TACGCATGCGGCAAGCATT TCATGTAGGAG-3' and 5'-ATTGTCGACGAGAGTCGGAATGTATCCTCA-3' [28]. These primers were designed to contain the *SphI* and *SalI* sites, respectively (shown by italic

letters). PCR was carried out with the following conditions: denaturation at 92 °C for 30 sec, annealing at 55 °C for 1 min, extension at 72 °C for 2 min; 30 cycles. The PCR fragment was digested with *Sph*I and *Sal*I, and cloned into the *Sph*I/*Sal*I site of pUC19 (pGR19). A 1.7 kb *Bam*HI fragment containing the *HIS3* gene was inserted between the *Cla*I and *Nhe*I sites internal to the *GLR1* gene in pGR19, to construct a plasmid pGRK3. Plasmid pGRK3 was digested with *Sph*I and *Sal*I, and a fragment containing *glr1::HIS3* was transformed to YPH250 to construct a strain GRK1 (*glr1Δ*). Disruption of each gene was verified by PCR. To construct the high copy number plasmid of the *ZWF1* gene, the following oligonucleotide primers were used in PCR to amplify a 2.1 kb fragment containing the open reading frame of the *ZWF1* gene from genomic DNA: 5'-TGGGGATCCCAAATGTCACTGACCGCGGC-3' and 5'-AAAGCATGCATAAGATGCATACTCCGCGCGG-3' [16]. The amplified DNA fragment was treated by Klenow fragment, and then cloned to *Bam*HI site of YEp13, which was also treated by Klenow fragment. The resultant plasmid was named YEpZWF1. The DNA fragment of the *GLR1* gene amplified by PCR was treated by Klenow fragment, then cloned into the *Bam*HI site of YEp13, which was also treated by Klenow fragment, to yield YEpGLR1. Transformation of yeast was performed by the method of electroporation (Bio-rad; Gene pulser II).

#### *H<sub>2</sub>O<sub>2</sub> treatment*

To observe the susceptibility to H<sub>2</sub>O<sub>2</sub>, cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial  $A_{610}$  = 0.1. This represented approximately 2 × 10<sup>6</sup> cells per ml. Various concentrations of H<sub>2</sub>O<sub>2</sub> were added to 5 ml samples, and cell survival was monitored by taking samples at 20 min intervals, diluting by the same buffer and plating aliquots on YPD agar plates. For adaptation experiments, cells were pretreated with resuspending in fresh YPD or SD medium containing a sublethal concentration of H<sub>2</sub>O<sub>2</sub> (0.2 mM) and incubated with shaking at 28 °C for 60 min. Pretreated cells were harvested and resuspended in 100 mM potassium phosphate buffer (pH 7.4), and challenged to the lethal concentration of H<sub>2</sub>O<sub>2</sub> (2 mM).

#### *Enzyme assays*

Cells were disrupted by vortexing with glass beads in 100 mM potassium phosphate buffer (pH 7.4) containing 1 mM PMSF and 1  $\mu$ g/ml pepstatin A. GR activity was determined by the method of Miki *et al.* [30]. G6PDH activity was determined by the method of Kuby and Notlman [31]. 6PGDH activity was determined by the method of Rippa and Signorini [32]. Protein was determined by the method of Lowry *et al.* [33].

#### *Determination of glutathione*

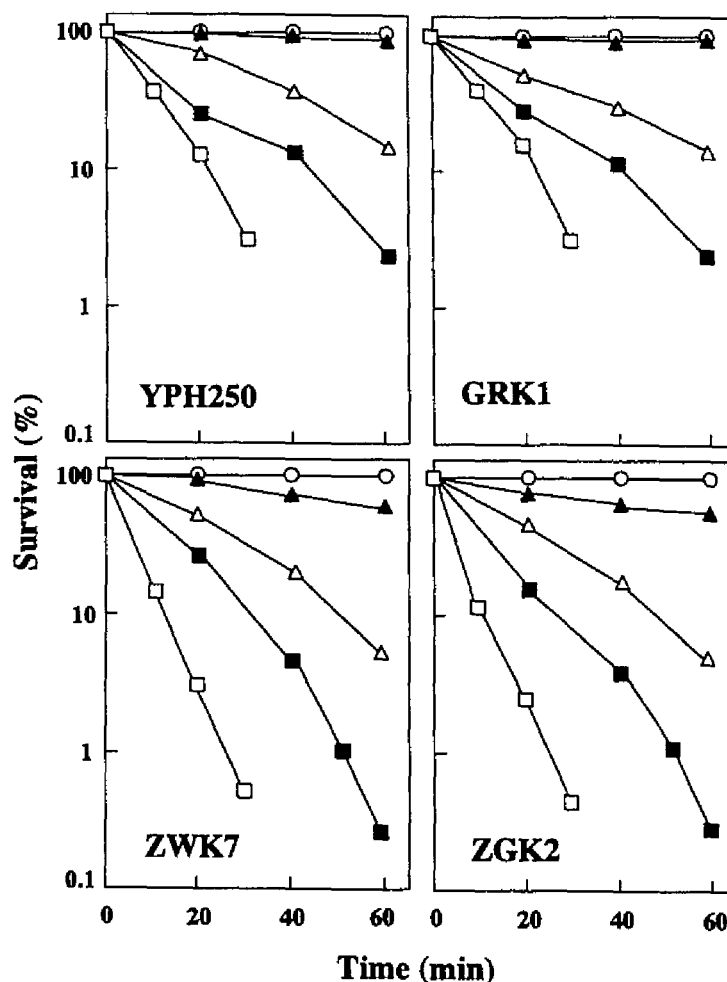
Total glutathione was determined by the method of Tietze [34], and glutathione disulfide was measured by the method of Anderson [35].

### **3-3. Results**

#### *Susceptibility to H<sub>2</sub>O<sub>2</sub> stress*

Wild-type and mutant cells in exponential growth phase were treated with various concentrations of H<sub>2</sub>O<sub>2</sub> (0.2-5 mM). G6PDH-deficient cells (ZWK7 and ZGK2) displayed greater susceptibilities to H<sub>2</sub>O<sub>2</sub> than wild-type cells (Figure 1). Juhnke *et al.* also reported that G6PDH-deficient mutant showed increased susceptibility to H<sub>2</sub>O<sub>2</sub> [17]. The author therefore confirms the importance of G6PDH in protection of *S. cerevisiae* against H<sub>2</sub>O<sub>2</sub> stress. In contrast, GR-deficient cells (GRK1) showed an almost identical susceptibility with that of wild-type cells. Grant *et al.* reported that there was no difference in susceptibility to H<sub>2</sub>O<sub>2</sub> between GR-deficient and wild-type cells during the exponential phase, but GR-deficient cells in the stationary phase were more sensitive than wild-type cells [11, 12]. We examined the susceptibility to H<sub>2</sub>O<sub>2</sub> using exponential-phase cells; therefore our data are consistent with the results of Grant *et al.*





**Fig. 1 Susceptibility of exponential-phase cells to  $H_2O_2$  stress**

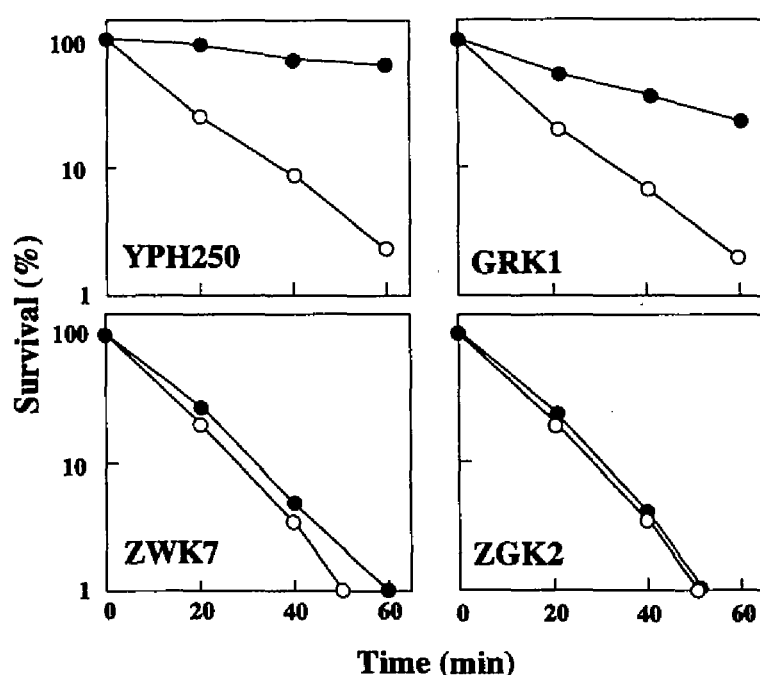
Cells growing exponentially in YPD medium were collected and resuspended in 100 mM potassium phosphate buffer (pH 7.4) to obtain the initial  $A_{610} = 0.1$  ( $2 \times 10^6$  cells  $ml^{-1}$ ), and treated with various concentrations of  $H_2O_2$ . Samples were diluted and plated on YPD agar plates to monitor the cell viability.  $H_2O_2$  concentrations were: 0 mM (○); 0.2 mM (▲); 1 mM (△); 2 mM (■); 5 mM (□). Results represent the mean from four independent experiments. Standard deviation did not exceed 5% in the range 100-10%, 1% in the range 10-1%, and 0.3% in the range 1-0.1%.

#### *Induction of adaptation to $H_2O_2$ stress*

We investigated the effects of G6PDH- and GR-deficiency on the induction of adaptation to  $H_2O_2$  stress. Adaptation was induced by the pretreatment of exponential-growth-phase cells with 0.2 mM  $H_2O_2$  for 60 min (first stress), and then the pretreated cells were challenged with 2 mM  $H_2O_2$  (second stress). With the 2 mM  $H_2O_2$  challenge, a difference in susceptibility was observed between wild-type cells and mutants (Figure 2). The pretreatment induced a large increase in tolerance to 2 mM  $H_2O_2$  in wild-type cells. In G6PDH-deficient cells

(ZWK7 and ZGK2), tolerance was hardly increased at all by the pretreatment, and adaptation was not induced. This result indicates that G6PDH is necessary for induction of adaptation to  $H_2O_2$  stress in *S. cerevisiae*.

GR-deficient cells, in spite of lacking GR activity, were still able to increase tolerance to  $H_2O_2$  stress, but not as efficiently as wild-type cells. This suggests that GR reaction is also important in adaptation. However, G6PDH is probably more important than GR for the adaptive response to  $H_2O_2$ .



**Fig. 2** Induction of adaptation to  $H_2O_2$  stress

Details of conditions for experiments are described in the text. ●, Cells were pretreated with 0.2 mM  $H_2O_2$  in YPD medium for 60 min, and then challenged to 2 mM  $H_2O_2$ . ○, Cells were challenged directly to 2 mM  $H_2O_2$ . Results represent the mean from four independent experiments. Standard deviation did not exceed 5% in the range 100-10%, 1% in the range 10-1%, and 0.3% in the range 1-0.1%.

#### *Changes in glutathione-recycling enzyme activities in the adaptive response*

We tried to clarify whether or not glutathione recycling affects induction of adaptation in G6PDH-deficient cells. Table 1 shows the changes in the enzyme activities involved in glutathione recycling (GR, G6PDH, and 6PGDH). In wild-type cells, the activity of each

enzyme was increased approximately 1.4 - 1.9-fold by the treatment with first stress (0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min). GR activity of the G6PDH-deficient mutants (ZWK7 and ZGK2) were somewhat higher than that of wild-type cells under non-stressed conditions, and was increased approximately 1.2-fold by the first stress treatment. 6PGDH activity of ZWK7 (*zwf1Δ*) was 55% lower than that of wild-type cells, and not increased by the first stress treatment. Comparing the G6PDH and 6PGDH activities of the GR-deficient mutant (GRK1) and the wild-type, 6PGDH activity was almost the same in the two strains, but G6PDH activity in GRK1 cells under non-stressed conditions was approximately 1.5-fold higher than that of wild-type cells. G6PDH and 6PGDH activities were increased approximately 1.2-fold and 1.5-fold by the first stress treatment even in the GR-deficient mutant.

**Table 1 Changes in glutathione-recycling enzyme activities in the adaptive response**  
Cells growing exponentially in YPD medium were collected, and resuspended in fresh YPD medium with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Values are means  $\pm$  S. E. M. from four independent experiments. ND, not detected. One unit (U) of 6PGDH activity is defined as the amount of enzyme oxidizing 1.0  $\mu$ mol of 6-phospho-D-gluconate/min at 25°C. Units for GR and G6PDH are defined in Table 2 in Chapter II.

Strain	H <sub>2</sub> O <sub>2</sub>	Activity (mU/mg-protein)		
		GR	G6PDH	6PGDH
YPH250	-	27.5 $\pm$ 1.1	451 $\pm$ 9	185 $\pm$ 25
	+	39.2 $\pm$ 4.2	861 $\pm$ 39	301 $\pm$ 75
ZWK7	-	34.2 $\pm$ 0.9	ND	84 $\pm$ 17
	+	42.2 $\pm$ 3.2	ND	82 $\pm$ 11
ZGK2	-	30.3 $\pm$ 1.0	ND	ND
	+	37.4 $\pm$ 2.9	ND	ND
GRK1	-	ND	691 $\pm$ 21	182 $\pm$ 41
	+	ND	814 $\pm$ 28	272 $\pm$ 63

#### *Changes in glutathione redox state in the adaptive response*

Table 2 shows the changes in the glutathione redox state in the adaptive response. Both total intracellular glutathione (tGSH) and oxidized glutathione (GSSG) in all strains showed the tendency to increase on exposure to the first stress. However, no significant alteration in the GSSG/tGSH ratio was observed after the treatment with the first stress. It seems that *S. cerevisiae* has the ability to keep the GSSG/tGSH ratio at a certain level under mild oxidative conditions. The levels of glutathione in the reduced form (tGSH—GSSG) in the mutants were slightly higher than that of the wild-type. GR-deficient cells contain a larger amount of GSSG than wild-type cells because of the lack of GR activity. The GSSG/tGSH ratio was

also higher in GR-deficient cells than wild-type cells (GRK1, 10.5; wild-type, 3.5). In G6PDH-deficient cells (ZWK7 and ZGK2), GR activity under non-stressed conditions was slightly higher than that of wild-type cells (Table 1), however, GSSG was not reduced as efficiently as in wild-type cells. The GSSG/tGSH ratio of these mutants were lower than that of GR-deficient cells, but higher than that of wild-type cells (ZWK7, 6.7; ZGK2, 6.3). Although both GSSG level and GSSG/tGSH ratio of GR-deficient cells were higher than those of G6PDH-deficient cells, GR-deficient cells still showed induction of adaptation and higher resistance against  $H_2O_2$  stress than G6PDH-deficient cells (Figures 1 and 2). These results indicate that increased susceptibility and inability to induce adaptation to  $H_2O_2$  stress in G6PDH-deficient cells were not caused by the insufficient recycling of glutathione alone.

**Table 2** Changes in glutathione redox state in the adaptive response

Cells growing exponentially in YPD medium were collected, and resuspended in fresh YPD medium with or without 0.2 mM  $H_2O_2$  for 60 min. Values are means  $\pm$  S. E. M. from four independent experiments.

Strain	$H_2O_2$	tGSH( $\mu$ mol/g)	GSSG(nmol/g)	GSSG/tGSH
YPH250	–	2.08 $\pm$ 0.03	73 $\pm$ 3	3.5
	+	2.25 $\pm$ 0.09	75 $\pm$ 4	3.3
ZWK7	–	2.47 $\pm$ 0.07	165 $\pm$ 4	6.7
	+	2.67 $\pm$ 0.08	175 $\pm$ 4	6.6
ZGK2	–	2.44 $\pm$ 0.06	153 $\pm$ 6	6.3
	+	2.51 $\pm$ 0.10	169 $\pm$ 3	6.7
GRK1	–	2.43 $\pm$ 0.04	254 $\pm$ 8	10.5
	+	2.69 $\pm$ 0.10	281 $\pm$ 5	10.4

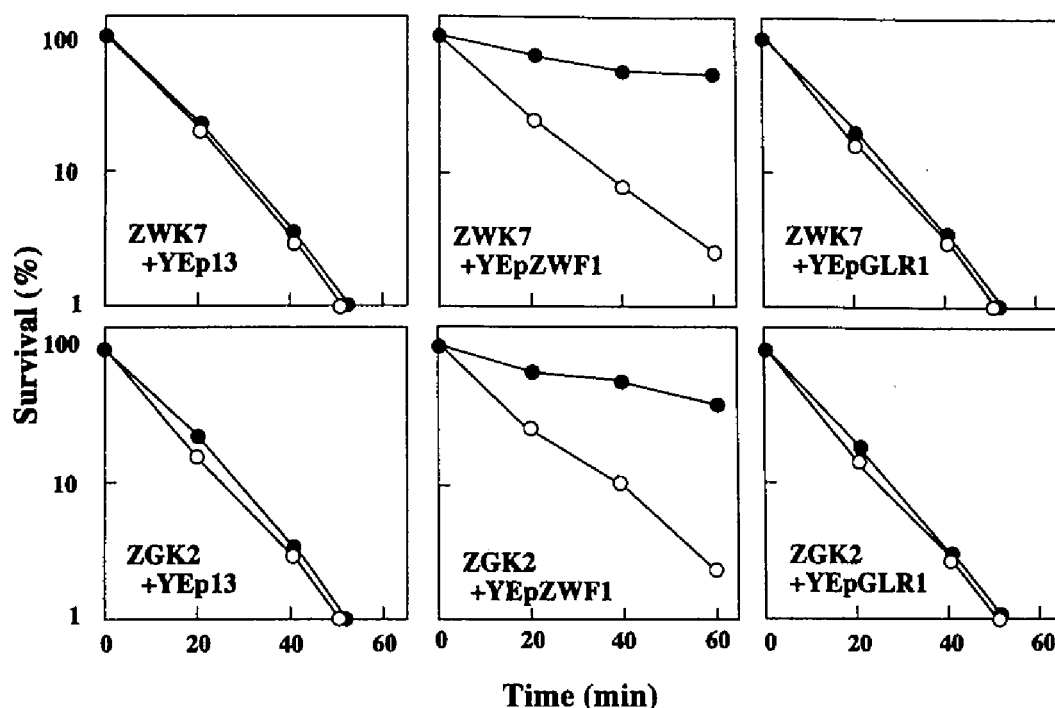
#### *Overexpression of the ZWF1 or GLR1 gene in G6PDH-deficient cells*

We next tested whether overexpression of the *ZWF1* or *GLR1* gene in G6PDH-deficient cells improves glutathione-redox state and reinstates the ability to induce adaptation to  $H_2O_2$  stress. Overexpression of the *ZWF1* gene in G6PDH-deficient cells (ZWK7+YEpZWF1 and ZGK2+YEpZWF1) rendered these mutants resistant to  $H_2O_2$  than the mutants carrying vector alone (ZWK7+YEp13 and ZGK2+YEp13). The induction of adaptation to 2 mM  $H_2O_2$  was recovered by overexpression of the *ZWF1* gene in G6PDH-deficient cells (Figure 3). In ZWK7+YEpZWF1 and ZGK2+YEpZWF1, G6PDH activities were approximately 2 - 3-fold higher than in wild-type cells under non-stressed conditions (Table 3). G6PDH activities in ZWK7+YEpZWF1 and ZGK2+YEpZWF1 were increased approximately 2-fold by  $H_2O_2$  treatment, as well as in the wild-type (Table 3). It has been reported that the *ZWF1* gene

contains a similar sequence to the yAP-1 recognition element in the 5'-flanking region [36]. The increase in G6PDH activity caused by the H<sub>2</sub>O<sub>2</sub> treatment may be due to induction of *ZWF1* gene expression under the control of yAP-1.

As observed in Table 3, 6PGDH activity was lowered in strain ZWK7 (*zwf1*Δ) carrying a vector YEp13 (ZWK7+YEp13, 50±13 mU/mg) or *GLR1*-overexpressing plasmid YEpGLR1 (ZWK7+YEpGLR1, 61±14 mU/mg) than in the wild-type (YPH250+YEp13, 177±16 mU/mg), however, surprisingly, 6PGDH activity increased approximately 8 - 9-fold by overproduction of G6PDH in ZWK7 (ZWK7+YEpZWF1, 461±32 mU/mg). 6PGDH activity was increased in proportion to the increase in G6PDH activity in wild-type cells and ZWK7+YEpZWF1, but not increased in G6PDH-deficient cells (ZWK7 and ZWK7+YEp13) under the stress conditions (Tables 1, 3). Sinha and Maitra reported that 6PGDH was induced by 6-phosphogluconolactone and 6-phosphogluconate in *S. cerevisiae* [37]. 6-Phosphogluconolactone is one of products of G6PDH reaction, and is hydrolyzed to 6-phosphogluconate by 6-phosphogluconolactonase. 6PGDH might be induced in yeast cells overexpressing the *ZWF1* gene because of a good supply of the substrate for the enzyme. In contrast, expression of the *GND1* gene encoding 6PGDH might be repressed in the *zwf1*Δ mutant because of insufficient supply of the substrate, 6-phosphogluconate. At any rate, a deficiency in G6PDH appears to affect the generation of NADPH via the pentose phosphate pathway.

In the case of GR activity, it was not affected significantly by the overexpression of the *ZWF1* gene. Both the quantity of GSSG and the GSSG/tGSH ratio were reduced to the level of the wild-type cells by overexpression of the *ZWF1* gene in G6PDH-deficient cells (Table 4). These results confirm that G6PDH is essential for efficient glutathione recycling and induction of adaptation.



**Fig. 3 Effect of the *ZWF1* or *GLR1* gene overexpression on the adaptation in G6PDH-deficient cells**

Details of conditions for the experiments are given in the text. ●, Cells were pretreated with 0.2 mM  $H_2O_2$  in SD medium for 60 min, and then challenged to 2 mM  $H_2O_2$ . ○, Cells were challenged directly with 2 mM  $H_2O_2$ . Results represent the average of four independent experiments. Standard deviation did not exceed 5% in the range 100-10%, 1% in the range 10-1%, and 0.3% in the range 1-0.1%.

On the other hand, overexpression of the *GLR1* gene in G6PDH-deficient cells (ZWK7+YEpGLR1 and ZGK2+YEpGLR1) did not allow these mutants to recover the ability to adapt to  $H_2O_2$  stress (Figure 3). Neither the quantity of GSSG nor the GSSG/tGSH ratio were reduced in ZWK7+YEpGLR1 and ZGK2+YEpGLR1, although these mutants had approximately 5-fold higher GR activity than that of wild-type cells and GR activity was increased by the  $H_2O_2$  treatment (Tables 3, 4). These results indicated that GR uncoupled from G6PDH may be unable to function efficiently to reduce GSSG.

**Table 3** Changes in glutathione-recycling enzyme activities in the adaptive response of G6PDH-deficient cells

Cells growing exponentially in SD medium were collected, and resuspended in fresh SD medium with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Values are means  $\pm$  S. E. M. from four independent experiments. Units are defined for each enzyme in Table 1.

Strain	H <sub>2</sub> O <sub>2</sub>	Activity (mU/mg-protein)		
		GR	G6PDH	6PGDH
ZWK7 + YEp13	–	27.5 $\pm$ 1.1	ND	50 $\pm$ 13
	+	39.2 $\pm$ 4.2	ND	58 $\pm$ 18
ZWK7 + YEpZWF1	–	34.2 $\pm$ 0.9	1344 $\pm$ 71	461 $\pm$ 32
	+	42.2 $\pm$ 3.2	2634 $\pm$ 47	640 $\pm$ 22
ZWK7 + YEpZWF1	–	116.3 $\pm$ 3.4	ND	61 $\pm$ 14
	+	173.2 $\pm$ 6.2	ND	70 $\pm$ 11
ZGK2 + YEp13	–	30.3 $\pm$ 1.0	ND	ND
	+	37.4 $\pm$ 2.9	ND	ND
ZGK7 + YEpZWF1	–	28.7 $\pm$ 3.0	912 $\pm$ 81	ND
	+	35.1 $\pm$ 3.4	1782 $\pm$ 98	ND
ZGK7 + YEpZWF1	–	108.3 $\pm$ 4.9	ND	ND
	+	161.3 $\pm$ 7.9	ND	ND
YPH250	–	24.9 $\pm$ 1.8	464 $\pm$ 7	197 $\pm$ 17
	+	37.5 $\pm$ 4.3	868 $\pm$ 32	259 $\pm$ 25
YPH250 + YEp13	–	22.6 $\pm$ 3.6	441 $\pm$ 15	177 $\pm$ 16
	+	36.4 $\pm$ 3.3	803 $\pm$ 27	244 $\pm$ 22

**Table 4** Changes in glutathione redox state in the adaptive response of G6PDH-deficient cells

Cells growing exponentially in SD medium were collected, and resuspended in fresh SD medium with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Values are means  $\pm$  S. E. M. from four independent experiments.

Strain	H <sub>2</sub> O <sub>2</sub>	tGSH( $\mu$ mol/g)	GSSG(nmol/g)	GSSG/tGSH
ZWK7 + YEp13	–	2.43 $\pm$ 0.04	154 $\pm$ 8	6.3
	+	2.69 $\pm$ 0.10	171 $\pm$ 5	6.4
ZWK7 + YEpZWF1	–	2.27 $\pm$ 0.07	85 $\pm$ 5	3.7
	+	2.57 $\pm$ 0.08	86 $\pm$ 3	3.3
ZWK7 + YEpZWF1	–	2.03 $\pm$ 0.11	164 $\pm$ 8	8.1
	+	2.21 $\pm$ 0.10	168 $\pm$ 7	7.6
ZGK2 + YEp13	–	2.57 $\pm$ 0.06	182 $\pm$ 10	7.1
	+	2.71 $\pm$ 0.05	188 $\pm$ 7	6.9
ZGK7 + YEpZWF1	–	2.44 $\pm$ 0.06	81 $\pm$ 7	3.3
	+	2.52 $\pm$ 0.05	83 $\pm$ 5	3.3
ZGK7 + YEpZWF1	–	2.11 $\pm$ 0.14	148 $\pm$ 8	7.0
	+	2.36 $\pm$ 0.12	156 $\pm$ 7	6.6
YPH250	–	2.12 $\pm$ 0.08	79 $\pm$ 4	3.7
	+	2.31 $\pm$ 0.09	72 $\pm$ 4	3.1
YPH250 + YEp13	–	2.06 $\pm$ 0.11	70 $\pm$ 6	3.5
	+	2.30 $\pm$ 0.07	78 $\pm$ 4	3.4

### 3-4. Discussion

The focus of this study was to evaluate whether insufficient recycling of glutathione, as the result of insufficient GR activity, is responsible for the increased susceptibility to  $H_2O_2$  stress of G6PDH-deficient yeast cells. To accomplish this objective, the author constructed G6PDH- and GR-deficient mutants and analyzed their phenotypes. G6PDH-deficient cells showed higher susceptibility to  $H_2O_2$  stress (Figure 1), and were unable to induce adaptation to such stress (Figure 2). Additionally, overexpression of the *ZWF1* gene in G6PDH-deficient cells reinstated the ability to induce adaptation to  $H_2O_2$  stress (Figure 3). G6PDH is the key enzyme to generate NADPH in the cytosol, and its activity could affect 6PGDH activity (Tables 1, 3). It has been reported that total NADPH level in G6PDH-deficient cells is reduced to 59-67% of that in isogenic wild-type [18]. These results indicate that the NADPH-generation by G6PDH is necessary for the acquisition of tolerance against  $H_2O_2$  stress in *S. cerevisiae*. It has been well known that G6PDH-deficiency in humans is characterized by an increased sensitivity to  $H_2O_2$  stress [22], and in some tissues (e.g., liver, adipose, lung and proliferating cells) G6PDH activity was increased by oxidative stress [14]. The author confirmed that G6PDH is necessary for the acquisition of tolerance against  $H_2O_2$  in *S. cerevisiae* as well as in humans.

It has been thought for a long time that the decrease in GSH, caused by loss of the NADPH-dependent activity of GR, is responsible for the increased susceptibility to oxidative stress of G6PDH-deficient animal cells [20-22]. In *S. cerevisiae* also G6PDH-deficiency resulted in insufficient reduction of GSSG. Even though sufficient amount of GR exists in G6PDH-deficient cells, both the amount of intracellular GSSG and GSSG/tGSH ratio were increased by the deficiency of G6PDH (Tables 1, 2). These results confirm the importance of G6PDH in the recycling of glutathione in *S. cerevisiae*. However, all mutants had amount of GSH that were equal to or higher than that of wild-type cells (Table 2). Therefore, the increased susceptibility and the inability to induce adaptation to  $H_2O_2$  in the G6PDH-deficient *S. cerevisiae* were not caused by decrease in intracellular GSH content.

The amount of GSSG and the GSSG/tGSH ratio of G6PDH-deficient cells were higher than those in wild-type cells, but lower than those in GR-deficient cells (Table 2). Adaptation was induced in GR-deficient cells, but not in G6PDH-deficient cells (Figure 2).



No correlation was found between the GSSG/tGSH and H<sub>2</sub>O<sub>2</sub> susceptibility, or between the GSSG/tGSH ratio and induction of adaptation to H<sub>2</sub>O<sub>2</sub> stress in *S. cerevisiae*. These results clearly demonstrate that incompleteness of glutathione recycling alone is not sufficient to explain the increased susceptibility and inability to induce adaptation to H<sub>2</sub>O<sub>2</sub> stress in G6PDH-deficient yeast cells. However, the author do not conclude that glutathione recycling is not important in the adaptive response in *S. cerevisiae*. Glutathione recycling must be necessary for the acquisition of the full increase of tolerance to H<sub>2</sub>O<sub>2</sub> stress, because GR-deficient cells were unable to increase the tolerance as efficiently as wild-type cells (Figure 2). In the adaptive response to H<sub>2</sub>O<sub>2</sub> stress, expression of the *GLR1* gene is induced by yAP-1 which is an oxidative stress responsible transcriptional activator [11, 12]. These data strongly suggest that glutathione recycling via the GR reaction must be one of the mechanisms of the adaptive response to H<sub>2</sub>O<sub>2</sub> stress.

It seems that GR contributes less than G6PDH to the increase in tolerance against H<sub>2</sub>O<sub>2</sub> in adaptation, since G6PDH deficiency showed more serious effects (Figures 1, 2). Excess GR in G6PDH-deficient cells had no effect on either glutathione-redox state or tolerance against H<sub>2</sub>O<sub>2</sub> (Figure 3, Table 4). Overproduction of GR in wild-type cells (YPH250+YEpGLR1) also had no effect on the glutathione-redox state and tolerance (data not shown). These results indicate that GR is unable to function at full capacity if not coupled to G6PDH, and that excess GR disproportionate with NADPH supply may be no use in reducing GSSG. It seems that the balance between GR activity and NADPH supply is important to recycle glutathione efficiently.

In GR-deficient cells, the GSSG/tGSH ratio did not change significantly with 0.2 mM H<sub>2</sub>O<sub>2</sub> treatment (Table 2). This result suggests that GSSG may be reduced by other thiol-disulfide oxidoreductases such as thioredoxin (TRX). Recently, Miller reported that GSSG was reduced by not only GR but also TRX in *S. cerevisiae* [38]. It has also been reported that GR is not required for maintenance of GSH in *E. coli* [25]. GR reaction may be compensated for or replaced to some extent by other mechanisms, such as TRX reaction or an increase in *de novo* synthesis of GSH, in the adaptive response to H<sub>2</sub>O<sub>2</sub> stress in *S. cerevisiae*.

On the other hand, G6PDH-deficiency affects cell metabolisms pleiotropically, since NADPH is involved in many enzyme reactions. For example, G6PDH-deficient *S. cerevisiae*

shows methionine auxotrophy [39]. Several studies have clearly demonstrated that mammalian and yeast catalases contain tightly bound NADPH and require NADPH to prevent the formation of compound II (inactive form) by  $\text{H}_2\text{O}_2$  [40–42]. Subsequently, Scott *et al.* have reported that the increased oxidant sensitivity of G6PDH-deficient erythrocytes correlated closely with NADPH levels and subsequent impaired catalase activity [22]. These data implied that the loss of catalase activity was the mechanism underlying the increased oxidant sensitivity of G6PDH-deficient erythrocytes. Yeast catalase also contains NADPH for prevention of the formation of compound II, and plays an important role in the acquisition of tolerance against oxidative stress by the adaptive response [29]. The loss of catalase activity may be one of mechanisms underlying the enhanced sensitivity of G6PDH-deficiency in *S. cerevisiae*, as well as in erythrocytes. In addition to catalase, TRX and thioredoxin peroxidase (TPx) would be influenced by G6PDH-deficiency. Oxidized TRX is reduced and recycled by NADPH-dependent reaction catalyzed by thioredoxin reductase. TRX is also important for the adaptive response to oxidative stress in *S. cerevisiae*. The *TRX2* gene, which encodes thioredoxin, is also regulated by yAP-1, and its expression is induced by  $\text{H}_2\text{O}_2$  stress [43]. Furthermore, TRX can act as an electron donor for TPx. TPx reduces  $\text{H}_2\text{O}_2$  and alkyl hydroperoxides, and serves as an antioxidant enzyme against oxidative stress in *S. cerevisiae* [44].

The decrease in NADPH level caused by G6PDH-deficiency affects many types of reactions in the adaptive response in *S. cerevisiae*, and glutathione recycling catalyzed by GR is just one of those reactions. Thus the mechanisms of the increased sensitivity and the inability to induce adaptation of G6PDH-deficient cells appear to be complicated. This implies that G6PDH plays an important role as the key enzyme in the adaptive response against oxidative stress. Schnell *et al.* have reported that G6PDH activity was increased by the overexpression of the *YAP1* gene, and that the *ZWF1* gene has the quite similar sequence of YRE (yAP-1 recognition element) in 5'-flanking region [36]. This suggests that the *ZWF1* gene may be regulated by yAP-1 at the transcriptional level in the oxidative stress response, as observed in the case of the *GSH1*, *GLR1*, and *TRX2* genes, and also implies that the induction of G6PDH is one of the mechanisms of adaptive response to  $\text{H}_2\text{O}_2$  in *S. cerevisiae*.

In conclusion, decrease of GSH content is not the reason for the increased sensitivity to  $\text{H}_2\text{O}_2$  in G6PDH-deficient *S. cerevisiae*. Glutathione recycling is one of the mechanisms of

the adaptive response to  $H_2O_2$ , and is certainly insufficient in G6PDH-deficient cells. Incompleteness of glutathione recycling appears to be one of the causes of increased sensitivity in G6PDH-deficient cells; however, this alone is not sufficient to explain the increased sensitivity and the inability to induce adaptation to  $H_2O_2$ . G6PDH must play other important roles in the adaptive response to  $H_2O_2$ , besides supplying NADPH to GR reaction. Further studies on the role of G6PDH in the adaptive response to oxidative stress are under way in order to clarify the function of G6PDH in *S. cerevisiae*.

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## Chapter 4

### Regulation of Yap1, an oxidative stress responsive transcription factor in *Saccharomyces cerevisiae*

#### 4-1. Introduction

Many aerobic organisms show adaptive responses to oxidative stress by increasing the levels of antioxidant enzymes. A portion of the adaptive response is regulated at the transcriptional level, and several transcription factors that regulate the expression of antioxidant genes have been reported. In *Escherichia coli*, OxyR, SoxR, and SoxS are key transcription factors for the adaptive response to oxidative stress. OxyR regulates the expression of genes encoding H<sub>2</sub>O<sub>2</sub>-inducible proteins, and SoxR and SoxS regulate the expression of genes encoding superoxide-inducible proteins [1]. In mammals, two transcription factors, NF- $\kappa$ B and AP-1, have been strongly implicated in the oxidative stress response [2]. The activities of these transcription factors are reversibly controlled through redox states and modulated by thiol-disulfide oxidoreductases such as thioredoxin (TRX) and glutaredoxin (GRX) [2, 3]. OxyR is activated through the formation of a disulfide bond between Cys<sup>199</sup> and Cys<sup>208</sup>, and deactivated by the enzymatic reduction of the bond with GRX [3]. The activities of NF- $\kappa$ B and AP-1 are also regulated by the redox modification of cysteine residues. After the dissociation of NF- $\kappa$ B/I $\kappa$ B complex, TRX augments the DNA-binding and transcriptional activities of NF- $\kappa$ B by reducing the Cys<sup>62</sup> residue in its DNA-binding loop [4-6]. Similarly, redox modification of AP-1 is regulated by a nuclear redox factor Ref-1, and the Ref-1 activity is also modulated by TRX [7, 8]. TRX can associate directly with Ref-1 in the nucleus [9, 10].

The contribution of TRX and GRX toward the maintenance of the intracellular environments in reduced states is comparable to glutathione [11, 12]. These thiol-disulfide oxidoreductases may participate in the control of redox-regulated transcription factors via the intracellular redox states [3, 13].

Yap1 (yeast AP-1) is a transcription factor crucial for oxidative stress response in *Saccharomyces cerevisiae*, and it regulates the expression of several genes whose gene products play major roles in the oxidative stress tolerance. The null mutant of the *YAP1* gene displayed hypersensitivity to oxidative stress and the overexpression of the *YAP1* gene confers

stress resistance [14-18]. Several target genes for Yap1 have been identified, such as *GSH1* encoding  $\gamma$ -glutamylcysteine synthetase [19], *GLR1* encoding glutathione reductase [20], *TRX2* encoding TRX [21], and *TRR1* encoding thioredoxin reductase [22]. These gene products are involved in the adaptive response to oxidative stress.

Yap1 was originally identified as a functional homologue of mammalian AP-1 on the basis of its ability to bind to an AP-1 recognition element [23]. Yap1 binds to the Yap1 recognition element (YRE: 5'-TTAGT(C/A)A-3') in the promoter region of target genes [24]. The N-terminus of Yap1 contains a bZip domain which is conserved among the AP-1 family, including mammalian Jun, Fos, and *S. cerevisiae* Gcn4 [23]. A cysteine rich domain (CRD) at the C-terminus of Yap1, containing three Cys-Ser-Glu sequence motifs, plays an important role in the control of Yap1, especially for the intracellular localization of Yap1 [25, 26]. In the response to oxidative stress, the localization of Yap1 changes dramatically, while the increase in the DNA-binding activity is modest and the levels of Yap1 do not increase [26-28]. Yap1 exists both in the cytoplasm and the nucleus under non-stressed conditions, while it is concentrated in the nucleus under oxidative conditions [26]. Recently, it has been reported that the localization of Yap1 is controlled by Crm1-mediated nuclear export and that Yap1 has an nuclear export sequence (NES) embedded within the CRD [29, 30]. Mutational analysis suggested that cysteine residues within the CRD serves as redox sensors which regulate the availability of the NES [30]. Therefore, removal of the CRD causes constitutive accumulation of Yap1 in the nucleus, which results in the increase of transcription of the Yap1-target genes [26]. Additionally, the CRD is required for Yap1 to discriminate the stresses elicited by H<sub>2</sub>O<sub>2</sub>, diamide, and CdCl<sub>2</sub> [27, 28].

The importance of the CRD raised the possibility that TRX and/or GRX modulate the activity of Yap1 by the redox modification of cysteine residues, like OxyR, NF- $\kappa$ B, and AP-1. To examine whether TRX and/or GRX attend the redox regulation of Yap1, we analyzed the phenotypes of the TRX- and GRX-deficient mutants with respect to the Yap1-mediated gene expression and the localization of Yap1. In this chapter, the author shows that TRX-deficiency (*trx1 $\Delta$ /trx2 $\Delta$* ) caused the constitutive activation of Yap1 under aerobic conditions but not under anaerobic conditions, and that GRX-deficiency (*grx1 $\Delta$ /grx2 $\Delta$* ) did not affect the Yap1 activity. The author also provides the evidence that the *yap1 $\Delta$ /trx1 $\Delta$ /trx2 $\Delta$*

triple mutant shows the synthetic lethality while the *yap1Δ/grx1Δ/grx2Δ* triple mutant was viable. Here it was investigated about the regulation of Yap1 and the roles of thioredoxin in the regulation.

## 4.2. Materials and Methods

### *Yeast Strains and Medium*

*S. cerevisiae* YPH250 and YPH252 were obtained from the Yeast Genetic Stock Center, University of California at Berkeley, USA. Cells were cultured in 50 ml SD minimal medium (2% glucose, 0.67% yeast nitrogen base w/o amino acids, pH 5.5) with appropriate amino acids and bases at 30 °C with reciprocal shaking in 200 ml Erlenmeyer flasks. For anaerobic cultivation, the medium was flushed with nitrogen gas, sealed up, and incubated without shaking at 30°C. Exponentially growing cells were harvested at  $A_{610} = 0.5$ .

### *Construction of TRX- and GRX-deficient Mutants*

The *yap1Δ* mutants were constructed by using pSM27 as described by Wu *et al.* [17]. pSM27 was digested with *EcoRI* and transformed to YPH250 (*MATa trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*) and YPH252 (*MATα trp-Δ1 his3-Δ200 lys2-801 leu2-Δ1 ade2-101 ura3-52*) to yield the *yap1Δ* and YA-1α, respectively. All mutants used in this study were derived from YPH250 except for YA-1α and YT-1α (see below).

*S. cerevisiae* has two cytosolic TRX genes, *TRX1* and *TRX2* [31], one mitochondrial TRX gene, *TRX3* [32], and two GRX genes, *GRX1* and *GRX2 (TTR1)* [33, 34]. To disrupt the *TRX1* gene, the *TRX1* gene was amplified using the following oligonucleotide primers: 5'-GATCAGAATGATTGAAATCA-3' and 5'-GACGAGCTATAGGATGATGA-3'. The amplicon (2.0 kb) was treated by Klenow fragment, and then cloned, respectively, to the *HincII* site of pUC19 (pTR-1) and to the *BamHI* site of YEpl3 which was also treated by Klenow fragment (YEplTRX1). The *URA3* gene (1.2 kb) isolated from YEpl24 was treated with Klenow fragment and inserted between the *MunI/MunI* sites internal to the *TRX1* gene in pTR-1, which was also treated by Klenow fragment, to yield pTRD1. pTRD1 was digested with *ApaLI* and *DraI*, and the *trx1Δ::URA3* fragment was transformed to YPH250 and YA-1α to yield the *trx1Δ* and YT-1α, respectively. Disruption of the *TRX2* gene was done



using the *trx2::HIS3* disruption plasmid kindly provided by Drs. S. Kuge and N. Jones [21]. The *trx2::HIS3* disruption plasmid was digested with the *SphI* and the *trx2Δ::HIS3* fragment was transformed to YPH250 and the *trx1Δ* mutant to yield the *trx2Δ* mutant and the *trx1Δ/trx2Δ* mutant, respectively. Additionally, the *trx2::LEU2* disruption plasmid was constructed. The *TRX2* gene was amplified by using the following oligonucleotide primers: 5'-GATCAGCATAACTTGAGTGC-3' and 5'-GATCGCATGGAACGCCAAGC-3'. The amplicon (0.8 kb) was treated with Klenow fragment, and then cloned to the *HincII* site of pUC19 (pTR-2), and to the *BamHI* site of YEp13 (YEpTRX2), respectively. The *LEU2* gene (2.2 kb) isolated from YEp13 was treated with Klenow fragment and inserted between the *HincII/EcoO65I* sites internal to the *TRX2* gene in pTR-2, which was also treated with Klenow fragment, to yield pTRD2. pTRD2 was digested with *PstI* and *SmaI* and the *trx2Δ::LEU2* fragment was transformed to the *yap1Δ* mutant to yield YT-2a.

Mitochondrial thioredoxin gene, *TRX3* (TCR083w), was also amplified using the following oligonucleotide primers: 5'-GGCGGAGAATAGGGATCCACTGCGA-3' and 5'-GTCTCCGCTGGATCCAGAATATAAC-3'. The amplicon (1.4 kb) was digested with *BamHI* and cloned to the *BamHI* site of YEp13 (YEpTRX3).

To disrupt the *GRX* genes, the *GRX1* gene was amplified using the following primers: 5'-CATCCTTAGAAAGGATCCCACATTG-3' and 5'-CGAGACGTACGGGATCCTAAAGTGG-3'. The amplicon (1.1 kb) was digested with *BamHI* and cloned to the *BamHI* site of YEp13 (YEpGRX1), and also to the *BamHI* site of pUC19 (pGR-1). A 1.2 kb *BglII-ClaI* fragment containing the *TRP1* gene from pRS414 was inserted between *BglII/ClaI* sites internal to the *GRX1* gene in pGR-1 (pGRD1). pGRD1 was digested with *SmaI* and *SalI* and the *grx1Δ::TRP1* fragment was transformed to YPH250 to yield the *grx1Δ* mutant. The *GRX2* gene was also amplified using the following primers: 5'-GGGTCATTGCCGTGGATCCTACAAAAC-3' and 5'-TACACGTGGATCCTGATGCTGAAGT-3'. The amplicon (1.2 kb) was digested with *BamHI* and cloned to the *BamHI* site of YEp13 (YEpGRX2), and also to the *BamHI* site of pUC19 (pGR-2). The *URA3* gene (1.2 kb) isolated from YEp24 was treated with Klenow fragment and inserted between the *BsaAI/BsaAI* sites internal to the *GRX2* gene (pGRD2). pGRD2 was digested with *SmaI* and *SacI* and the *grx2Δ::URA3* fragment was transformed to YPH250 and the *grx1Δ* to yield the *grx2Δ* mutant and the

*grx1Δ/grx2Δ* mutant, respectively. Disruption of each gene was verified by PCR. The *trx1Δ/trx2Δ* mutant showed the methionine auxotrophy, the increase of cell size, and elongation of generation time as reported by Muller [35].

#### *Construction of the GSH1-lacZ Fusion Gene*

The *GSH1* promoter fragment containing the region from -800 to +33 (+1 representing the start of translation) was generated by the PCR using the primers: 5'-TAATCTTATGAATCCCGGGGATTTTATCGG-3' and 5'-CTAGACTCAAACCCGGGCA AAGGCGTGCCC-3'. The amplicon was digested with *Sma*I and cloned into the *Sma*I site of pMC1871 containing the coding region of *lacZ* (Amersham Pharmacia Biotech Ltd.), to yield pMC-GSH1lacZ. As a result of this construction, first 11 amino acid residues of Gsh1 was fused to  $\beta$ -galactosidase whose first 7 amino acids were deleted. The *GSH1-lacZ* in pMC-GSH1lacZ was isolated by digestion with *Sal*I and cloned into the *Sal*I site of pRS414 (pRS-GSH1lacZ).  $\beta$ -Galactosidase activity was measured as described by Miller [36]. One unit of the activity was defined as the amount of enzyme increasing  $A_{420}$  per hour at 30°C. Protein was determined by the method of Lowry *et al.* [37].

#### *Northern Blotting Analysis*

Northern hybridization was performed using 25  $\mu$ g of total cellular RNA isolated from yeast cells by the method of Schmitt *et al.* [38]. The probes were generated by random primed labeling of the 0.4 kb *Eco*RV-*Bam*HI fragment of *GSH1* gene, 0.7 kb *Pvu*II-*Hinc*II fragment of *TRR1* gene, and 1.1 kb *Eco*RI-*Pst*I fragment of *ACT1* gene, respectively, with [ $\alpha$ -<sup>32</sup>P]dCTP using a kit (Random Primer DNA Labeling Kit Ver.2, Takara).

#### *GFP-Yap1 Fusion*

pRS cup1 cp-GFP-YAP1 and the cysteine substitution mutants of Yap1-GFP fusion were kindly gifted by Dr. Kuge [26]. To visualize DNA, the cells were stained with 1  $\mu$ g/ml 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI).

#### *Production of Anti-TRX antibody*

A *NdeI/BamHI* fragment encoding the whole coding region of the *TRX2* gene was inserted into the *NdeI/BamHI* site of pET15-b (Novagen) to construct pET-TRX2. The resulting protein contained six histidine-tag residues fused in-frame with the *TRX2* coding region. pET-TRX2 was transferred into *E. coli* strain BL21(DE3). Histidine-tagged Trx2 was purified using the histidine-affinity column chromatography (His-trap, Pharmacia) followed by gel filtration chromatography (Superdex 75, Pharmacia). Purified fusion protein was then used for the production of anti-yeast TRX antibody using New Zealand White rabbits. Immunization and purification of anti-Trx antibody were accomplished by Sawady Technology Co., Ltd., Tokyo, Japan. This anti-TRX antibody was specific to yeast TRX, and was able to detect both Trx1 and Trx2.

#### *Immunofluorescence technique*

Immunofluorescence microscopic observation of yeast was performed by the methods of Rose *et al.* [39]. Anti-rabbit IgG (H+L)-FITC (FI-1000, Vector lab. Inc.) was used as the secondary antibody.

#### *Western Blotting Analysis*

Cell extracts were prepared according to Wemmie *et al.* [28], and 170  $\mu$ g of protein of each sample was run on 10% SDS-polyacrylamide gels. Proteins were electrically transferred to PVDF membrane (Immobilon, Millipore). Anti-Yap1 antiserum raised in rabbit was kindly gifted by Dr. Moye-Rowley. Horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research Lab., Inc.) and diaminobenzidine were used to visualize immunoreactive protein.

#### *Glutathione Reductase, Total Glutathione, and H<sub>2</sub>O<sub>2</sub> Stress Treatment*

The activity of glutathione reductase, total glutathione, and the susceptibility and adaptation to H<sub>2</sub>O<sub>2</sub> were determined as described previously [40, 41].

#### *Measurement of Intracellular Oxidation*

Intracellular oxidation level of yeast was measured using the oxidant-sensitive probe 2',

7'-dichlorofluorescein diacetate (DCFH-DA) purchased from Molecular Probes [42]. Cells growing exponentially in SD medium was collected and resuspended in fresh SD medium containing 0.1 mM DCFH-DA and incubated at 30°C for 20 min. After the incubation, cells were washed, resuspended in distilled water, and disrupted by vortexing with glass beads. Cell extracts (70  $\mu$ l) were mixed in 500  $\mu$ l distilled water, and fluorescence was measured with  $\lambda_{\text{EX}} = 490$  nm and  $\lambda_{\text{EM}} = 524$  nm using a Hitachi F-3000 spectrofluorometer.

#### *Genetic Manipulation of Yeast*

Mating, sporulation, dissection, and tetrad analysis of yeast were done as described by Rose *et al.* [39].

### **4.3. Results**

#### *The TRX-deficient Mutant Shows the Constitutive Activation of Yap1*

In order to see the effect of TRX and GRX on the activity of Yap1, we investigated the levels of cellular glutathione and glutathione reductase (GR) activity in the TRX- and GRX-deficient mutants. The *GSH1* and *GLR1* genes, which are target genes for Yap1 (19, 20), encode  $\gamma$ -glutamylcysteine synthetase (Gsh1) and GR, respectively [43, 44]. The Gsh1 catalyzes the first and rate limiting step of glutathione synthesis [45]. Therefore, it is conceivable that the levels of intracellular glutathione and GR activity reflect the activity of Yap1.

Table I shows the levels of total glutathione and GR activity in various mutants. As reported previously, the *yap1* $\Delta$  mutant showed lower levels of total glutathione and GR activity than the wild-type [19, 20]. Total glutathione and GR activity in the wild-type were increased by the treatment with 0.2 mM H<sub>2</sub>O<sub>2</sub>, but not in the *yap1* $\Delta$  mutant [46]. The levels of glutathione and GR activity in *trx1* $\Delta$ /*trx2* $\Delta$  were constitutively high under non-stressed conditions, and the values were higher than those in the wild-type which was treated with H<sub>2</sub>O<sub>2</sub>. This indicates that the loss of both Trx1 and Trx2 affects intracellular glutathione metabolism. GRX-deficiency (*grx1* $\Delta$ /*grx2* $\Delta$ ) and single-gene mutation of TRX or GRX genes did not affect total glutathione levels and GR activity (data for single-gene mutants are not shown), and our results are consistent with the previous reports [34, 47]. The results in Table I imply that Yap1 is constitutively activated in *trx1* $\Delta$ /*trx2* $\Delta$  even though the cells are not

exposed to oxidative stress.

To confirm the constitutive activation of Yap1 in the TRX-deficient mutant, we next assessed the expression of the *lacZ* reporter gene under the control of the *GSH1* promoter. A fusion gene was constructed containing the *lacZ* coding region fused to the *GSH1* promoter sequence which contains the Yap1 binding site (5'-TTAGTCA-3') (19). As shown in Figure 1A,  $\beta$ -galactosidase activity was increased with H<sub>2</sub>O<sub>2</sub> treatment in the wild-type, but not in the *yap1* $\Delta$ , as reported previously [48]. The  $\beta$ -galactosidase activities in single mutants (*trx1* $\Delta$  or *trx2* $\Delta$ ) under non-stressed conditions were the same to that in the wild-type. On the contrary, the basal level of  $\beta$ -galactosidase activity in *trx1* $\Delta$ /*trx2* $\Delta$  was significantly higher than that of the wild-type, and the value was almost the same as that of the wild-type treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> (the wild-type with H<sub>2</sub>O<sub>2</sub>, 88.8  $\pm$  1.3; *trx1* $\Delta$ /*trx2* $\Delta$  w/o H<sub>2</sub>O<sub>2</sub>, 95.1  $\pm$  1.2 U/mg). The  $\beta$ -galactosidase activity in *trx1* $\Delta$ /*trx2* $\Delta$  was scarcely increased by the treatment with H<sub>2</sub>O<sub>2</sub>, suggesting that Yap1 activity was constitutively high and presumably saturated in the *trx1* $\Delta$ /*trx2* $\Delta$  mutant under non-stressed conditions.

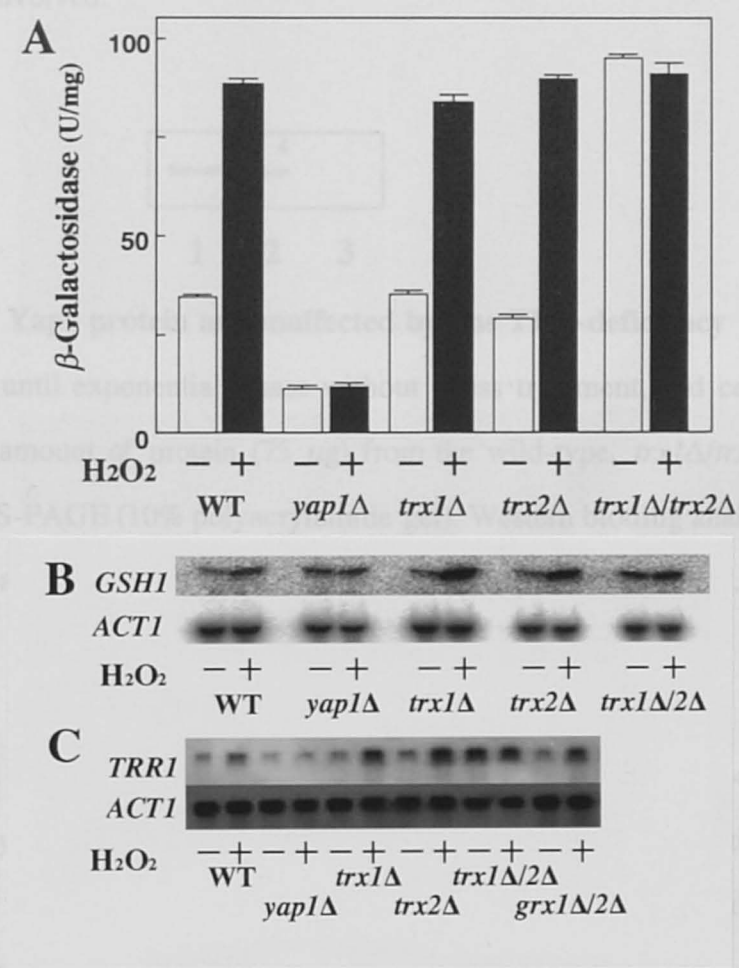
**Table 1 Cellular total glutathione contents and GR activity in various mutants**

Cells growing exponentially were treated with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min.

Data are means  $\pm$  SD from three independent experiments. One unit of GR was defined as the amount of enzyme reducing 1.0  $\mu$ mol of GSSG per min at 25 °C.

Strain	H <sub>2</sub> O <sub>2</sub>	Glutathione ( $\mu$ mol/g-cell)	GR activity (mU/mg)
WT	–	1.81 $\pm$ 0.12	74.2 $\pm$ 5.8
	+	2.08 $\pm$ 0.11	97.3 $\pm$ 6.4
<i>yap1</i> $\Delta$	–	0.85 $\pm$ 0.09	46.5 $\pm$ 4.2
	+	0.87 $\pm$ 0.09	51.0 $\pm$ 5.0
<i>grx1</i> $\Delta$ / <i>grx2</i> $\Delta$	–	1.76 $\pm$ 0.12	76.0 $\pm$ 5.4
	+	1.90 $\pm$ 0.12	95.4 $\pm$ 3.8
<i>trx1</i> $\Delta$ / <i>trx2</i> $\Delta$	–	2.78 $\pm$ 0.22	117.0 $\pm$ 8.4
	+	3.14 $\pm$ 0.20	143.0 $\pm$ 8.8

*Yap1* We reconfirmed the increased expression of the *GSH1* gene in *trx1Δ/trx2Δ* by Northern blotting analysis (Figure 1B). Basal level of the *GSH1* gene expression in *trx1Δ/trx2Δ* was almost the same as that of the H<sub>2</sub>O<sub>2</sub>-treated wild-type. Expression of the *GSH1* gene in *trx1Δ/trx2Δ* did not increase with 0.2 mM H<sub>2</sub>O<sub>2</sub> stress. These results were in good agreement with those obtained by using the *GSH1-lacZ* reporter gene assay. Expression of the *TRR1* gene, another target gene for Yap1 [22], was also constitutively high in *trx1Δ/trx2Δ*, but not in *grx1Δ/grx2Δ* (Figure 1C). Both experiments showed that the deficiency of TRX (*trx1Δ/trx2Δ*) resulted in constitutive acceleration of the expression of Yap1-target genes even under non-stressed conditions.

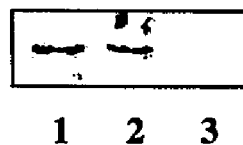


**Fig. 1 Effect of the TRX-deficiency on the expression of Yap1-target genes**

(A) Cells at exponential phase were harvested, resuspended in fresh SD medium and treated with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Cell extracts were prepared and  $\beta$ -galactosidase activity (*GSH1-lacZ*) was assayed. Data are means  $\pm$  SD from three independent experiments. (B, C) Cells were treated with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. Total cellular RNA was prepared, and 25  $\mu$ g of RNA was applied to each slot.

### *Yap1 Is Constitutively Localized in the Nucleus in $trx1\Delta/trx2\Delta$*

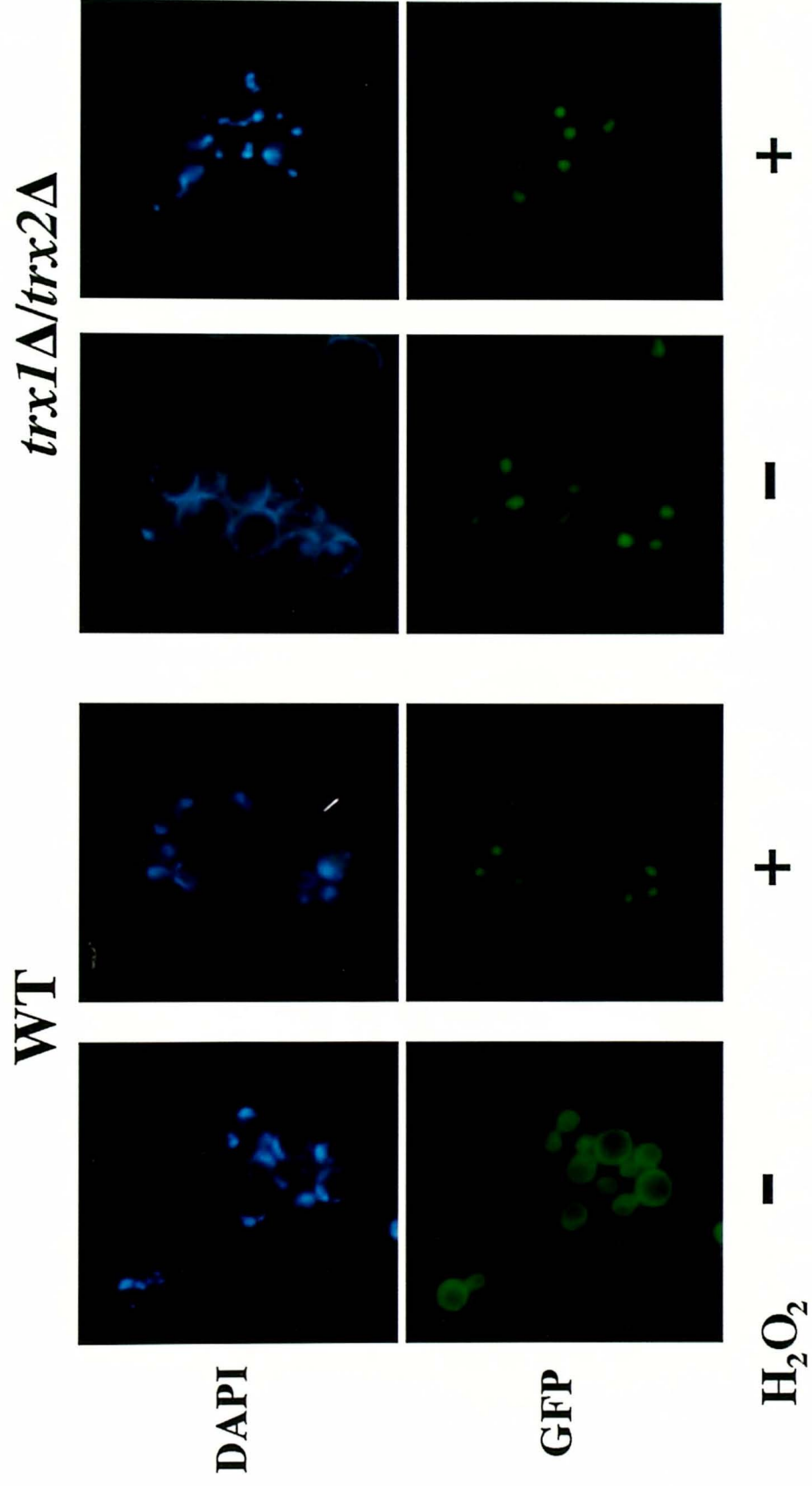
To determine whether constitutive high expression of Yap1-target genes is caused by the increase of Yap1 protein or not, we compared the amounts of Yap1 in the wild-type and  $trx1\Delta/trx2\Delta$  under non-stressed conditions by Western blotting analysis. The Yap1-specific bands were detected at similar intensities both in the wild-type and the  $trx1\Delta/trx2\Delta$  mutant (Figure 2). With the consideration of previous reports that the levels of Yap1 do not change during oxidative stress [27, 28], the constitutive high activity of Yap1 in  $trx1\Delta/trx2\Delta$  under non-stressed conditions can be attributed to a post-translational modification of Yap1, where TRX is likely to be involved.



**Fig. 2 The levels of Yap1 protein are unaffected by the TRX-deficiency**

Cells were cultured until exponential phase without stress treatment, and cell extracts were prepared. An equal amount of protein (75  $\mu$ g) from the wild-type,  $trx1\Delta/trx2\Delta$ , and  $yap1\Delta$  was subjected to SDS-PAGE (10% polyacrylamide gel). Western blotting analysis was carried out using anti-Yap1 antiserum. *Lanes: 1, wild-type; 2,  $trx1\Delta/trx2\Delta$ ; 3,  $yap1\Delta$ .*

It is reported that the Yap1 activity is regulated at the level of its localization. Yap1 exists both in the cytoplasm and in the nucleus under non-stressed conditions, while it is concentrated in the nucleus by oxidative stress [26]. We reconfirmed this by using the GFP-Yap1 fusion in the wild-type (Figure 3). In a single mutant of  $trx1\Delta$  or  $trx2\Delta$ , Yap1 behaved similarly to that in the wild-type (data not shown). On the other hand, Yap1 was constitutively localized in the nucleus in  $trx1\Delta/trx2\Delta$  not only under oxidative-stressed conditions but also under non-stressed conditions. Highly constitutive expression of the Yap1-target genes in  $trx1\Delta/trx2\Delta$  was most likely caused by the constitutive localization of Yap1 in the nucleus.



**Fig. 3**



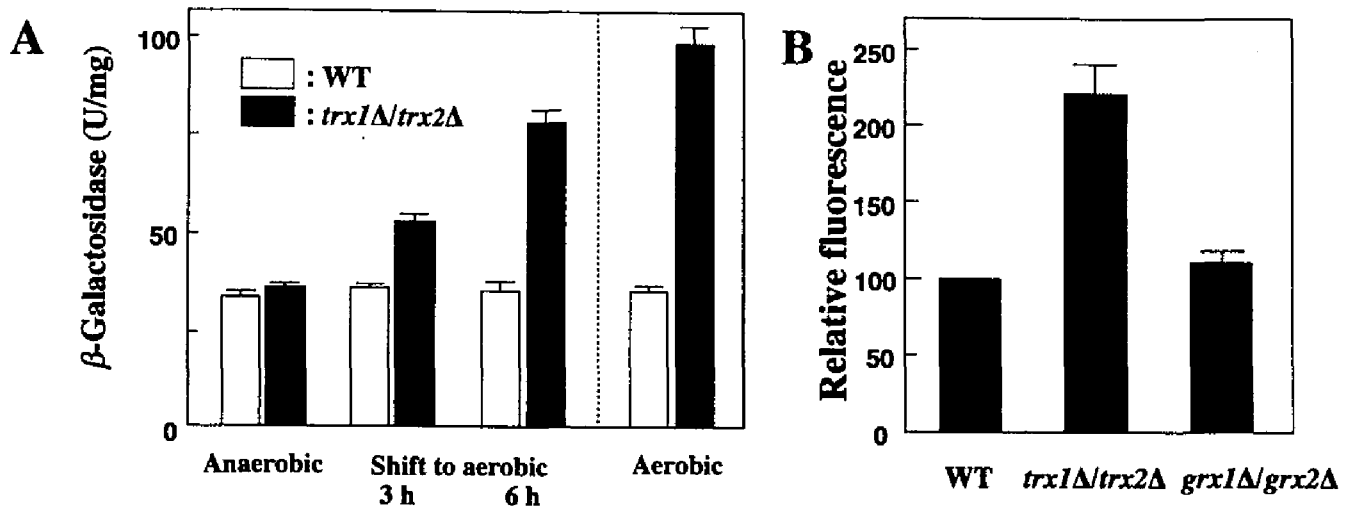
**Fig. 3 Yap1 is constitutively localized in the nucleus in the *trx1Δ/trx2Δ* mutant**

GFP fluorescence was visualized in living yeast cells carrying pRS cp-GFP-YAP1 (25). Cells at exponential phase were harvested, resuspended in fresh SD medium and treated with or without 0.2 mM H<sub>2</sub>O<sub>2</sub>. Cells were stained with DAPI (1 μg/ml) to visualize DNA.

*High Activity of Yap1 in *trx1Δ/trx2Δ* Was Repressed under Anaerobic Conditions*

It has been reported that cysteine residues are important in the redox-regulation of AP-1, NF-κB, and OxyR [2, 3]. Yap1 also contains a cysteine rich domain (CRD) at the C-terminus, and it was reported that cysteine residue(s) within the CRD is necessary to mediate the response to oxidative stress [26-28, 30]. Thus, Yap1 also might be regulated via the redox-modification of cysteine residue(s) and TRX may participate in this redox-regulation. To assess this, we investigated the activity of Yap1 in the *trx1Δ/trx2Δ* mutant under anaerobic conditions. β-Galactosidase activity derived from *GSH1-lacZ* fusion in the wild-type was virtually the same under both anaerobic and aerobic conditions without oxidative stress. On the other hand, β-galactosidase activity in *trx1Δ/trx2Δ* was markedly repressed under anaerobic conditions to the level similar to the wild-type (Figure 4A). Furthermore, this repression was restored by the transfer of the cells to the aerobic conditions. These results strongly suggest that redox states in the cells reflect the activity of Yap1 in *trx1Δ/trx2Δ*.

Additionally, we measured the levels of intracellular oxidation in *trx1Δ/trx2Δ* using the oxidant-sensitive probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). The level of intracellular oxidation in *trx1Δ/trx2Δ* was more than 2-fold higher than that in the wild-type, while that in *grx1Δ/grx2Δ* was only 1.1-fold higher (Figure 4B). These results indicate that the intracellular environments of *trx1Δ/trx2Δ* were in more oxidized states than those of the wild-type. Redox states of the intracellular environments and the redox regulation of transcription factors are suggested to be well linked [3, 13]. Thus the oxidative environments in *trx1Δ/trx2Δ* may also account for the constitutive activation of Yap1.



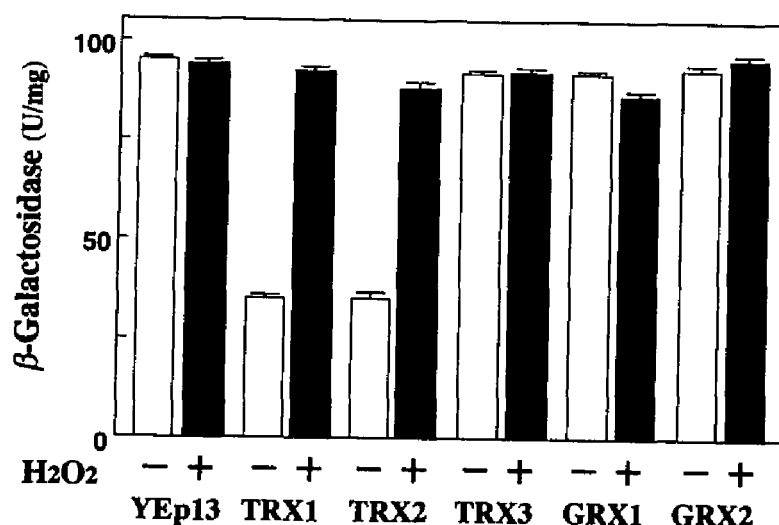
**Fig. 4 The activity of Yap1 is repressed under anaerobic conditions in *trx1Δ/trx2Δ***

(A) Wild-type and *trx1Δ/trx2Δ* cells were cultured until exponential phase without shaking under anaerobic conditions. Cells were then harvested and resuspended in fresh SD medium and incubated with shaking under aerobic conditions for another 3 or 6 hours at 30°C. Cell extracts were prepared and  $\beta$ -galactosidase activity (*GSH1-lacZ*) was assayed as described in the text. For control experiments, cells were grown with shaking under aerobic conditions. (B) Cells at exponential phase were harvested and resuspended in fresh SD medium containing 0.1 mM DCFH-DA for 20 min at 30°C, and then cell extracts were prepared. Fluorescence intensity was measured with  $\lambda_{EX}=490$  nm and  $\lambda_{EM}=524$  nm. Fluorescence intensity of the wild-type are relatively taken as 100. Data are means  $\pm$  SD from three independent experiments.

#### *GRX Does Not Substitute for TRX*

The constitutive activation of Yap1 was observed only in the TRX-deficient mutant (*trx1Δ/trx2Δ*), but not in the GRX-deficient mutant (*grx1Δ/grx2Δ*). However, in *E. coli*, the thioredoxin system and the glutaredoxin system can partially substitute for each other *in vivo* [11, 12]. Overexpression of the *grxA* gene in a *trxA* mutant can substitute for thioredoxin 1 in *E. coli* [49]. We examined whether or not the overexpression of GRX gene in *trx1Δ/trx2Δ* can repress the constitutive activation of Yap1. The Yap1 activity in *trx1Δ/trx2Δ* was reduced

to the basal level of the wild-type by overexpression of either the *TRX1* or *TRX2* gene alone; i.e., the levels of total glutathione, GR activity, and the expression of *GSH1-lacZ* were reduced to those of the wild-type (Figure 5, data of total glutathione and GR activity are not shown). On the other hand, overexpression of the *GRX1* or *GRX2* gene did not affect the Yap1 activity in *trx1Δ/trx2Δ*. Therefore, GRX was thought to be unable to substitute for TRX in the regulation of Yap1 activity *in vivo*. Overexpression of *TRX3* gene encoding mitochondrial TRX also did not affect the Yap1 activity in *trx1Δ/trx2Δ*, suggesting the difference of functions between cytosolic TRX and mitochondrial TRX.



**Fig. 5 GRX does not affect the activity of Yap1**

Cells of the *trx1Δ/trx2Δ* mutant carrying YEp13-based plasmids (YEpTRX1, YEpTRX2, YEpTRX3, YEpGRX1, YEpGRX2) were cultured until exponential phase, and treated with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 60 min. Cell extracts were prepared and  $\beta$ -galactosidase activity (*GSH1-lacZ*) was assayed as described in the text. Data are means  $\pm$  SD from three independent experiments.

#### *Cysteine-residues in the CRD are important for the regulation of Yap1*

The activity of Yap1 was affected by the redox conditions (Figure 4A) indicating that Yap1 also may be regulated via the redox states of cysteine residues likewise NF- $\kappa$ B or OxyR [3-6]. Therefore, the author estimated the roles of cysteine residues of Yap1 using the cysteine substitution mutants Yap1-GFP fusion [26]. The cm3-Yap1, all three cysteine residues in the transcriptional activation domain were substituted with threonine residues, showed the

similar intracellular localization with the wild-type Yap1 in the *trx1Δ/trx2Δ* mutant (Figure 6). This result indicates that these three cysteine residues do not take part in the regulation of Yap1 via the redox conditions.

On the other hand, the cm46A5-Yap1 never concentrated into the nucleus in the *trx1Δ/trx2Δ* mutant (Figure 7). This results strongly indicates that the thioredoxin-deficiency affects these cysteine residues. It seems that the thioredoxin-deficiency affects the redox states of these cysteine residues near the NES in the CRD, and leads the constitutive accumulation of Yap1 in the nucleus (Figure 3). Furthermore, this mutant did not concentrate into the nucleus by the treatment with 0.2 mM H<sub>2</sub>O<sub>2</sub> in the wild-type cells (Figure 8), indicating that the redox states of these cysteine residues regulates the nuclear export of Yap1 in the stress response.

**Fig. 6 The intracellular localization of cm3-Yap1 in *trx1Δ/trx2Δ* under non-stressed conditions**

Cells of the *trx1Δ/trx2Δ* mutant carrying GFP-cm3-Yap1 were cultured until exponential phase without stress. cm3-Yap1 is a cysteine substitution mutants of Yap1: Cys<sup>303</sup>, Cys<sup>310</sup>, and Cys<sup>315</sup> were substituted with threonine.

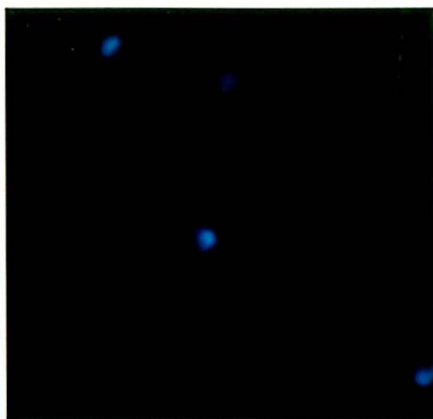
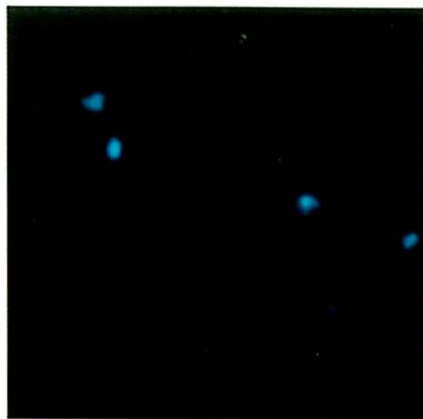
**Fig. 7 The intracellular localization of cm46A5-Yap1 in *trx1Δ/trx2Δ* under non-stressed conditions**

Cells of the *trx1Δ/trx2Δ* mutant carrying GFP-cm46A5-Yap1 were cultured until exponential phase without stress. cm46A5-Yap1 is a cysteine substitution mutants of Yap1: Cys<sup>598</sup> and Cys<sup>629</sup> were substituted with threonine, and Cys<sup>620</sup> was substituted with alanine.

**Fig. 8 The intracellular localization of cm46A5-Yap1 in the wild-type cells**

Cells of the wild-type YPH250 carrying GFP-cm46A5-Yap1 were cultured until exponential phase, and treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> in the fresh SD medium.

DAPI



GFP



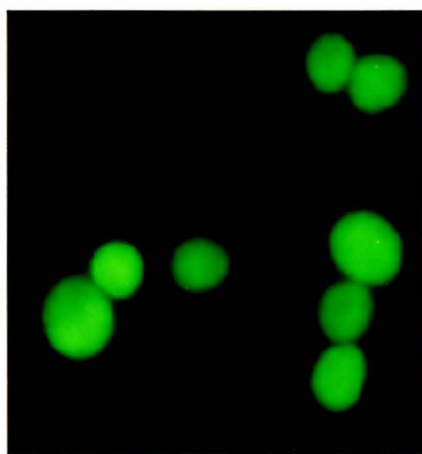
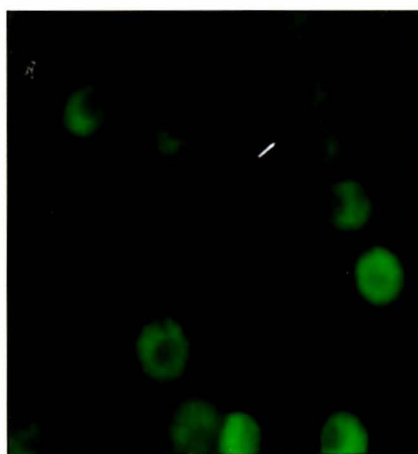
H<sub>2</sub>O<sub>2</sub>

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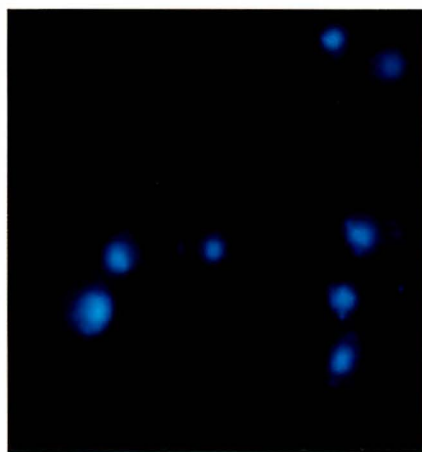
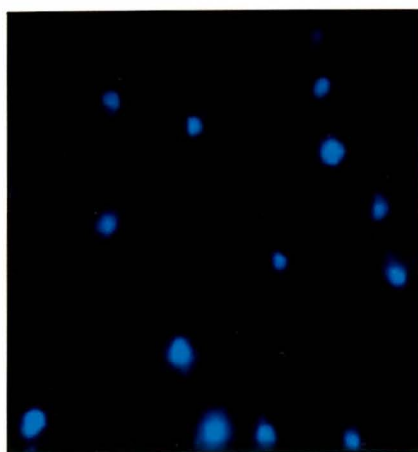
+

Fig. 8

TRXs



DAPI



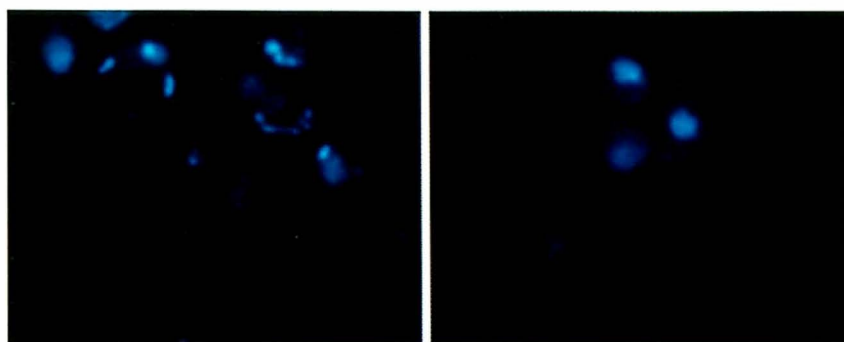
H<sub>2</sub>O<sub>2</sub>

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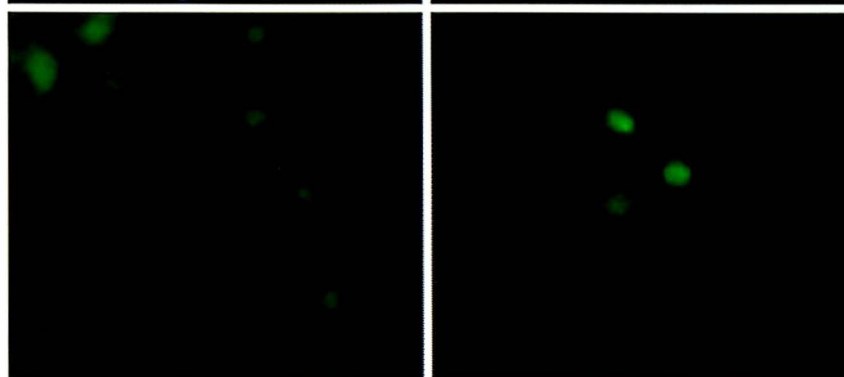
+

Fig. 9

**DAPI**



**GFP**

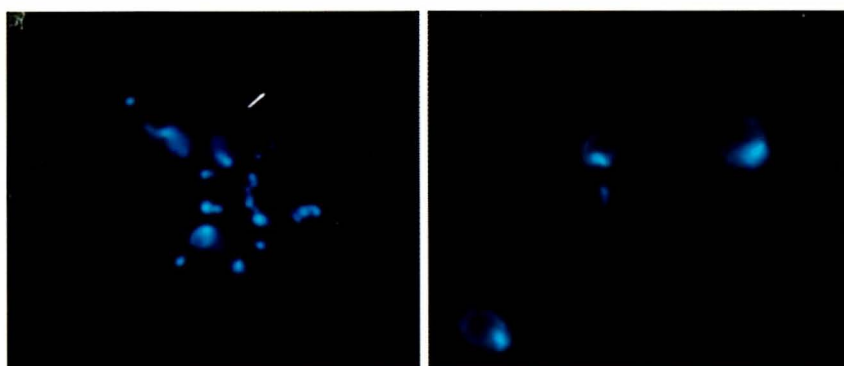


**WT-Yap1**

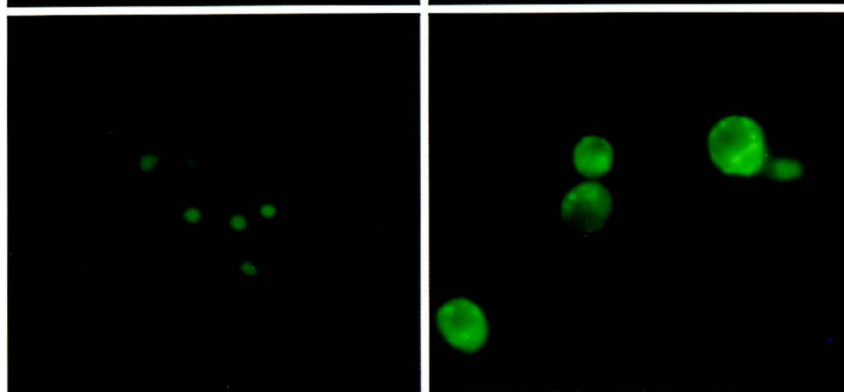
**cm 3-Yap1**

**Fig. 6**

**DAPI**



**GFP**



**WT-Yap1**

**cm46A5Yap1**

**Fig. 7**

### *Interaction between TRX and Yap1*

The constitutive activation of Yap1 in *trx1Δ/trx2Δ* suggests that TRX may regulate the Yap1 activity negatively. To see whether TRX associates with Yap1 directly or not, we first investigated the intracellular localization of TRX by immunofluorescence microscopy using anti-TRX antibody. If TRX interacts with Yap1 directly to repress the Yap1 activity, the intracellular localization of TRX is expected to be similar to that of Yap1. Yeast TRX would be able to enter or leave the nucleus by diffusion through the nuclear pore complex on account of its molecular size (molecular weights of both Trx1 and Trx2 are approximately 11 kDa) [50]. TRX was observed predominantly in the cytoplasm under non-stressed conditions (Figure 9). After the treatment of the wild-type cells with a mild oxidative stress (0.2 mM H<sub>2</sub>O<sub>2</sub>), TRX was observed both in the cytoplasm and in the nucleus, suggesting the possibility that TRX can associate with Yap1 both in the nucleus and in the cytoplasm. The increase of the levels of TRX protein by H<sub>2</sub>O<sub>2</sub> (0.2 mM) treatment was also confirmed by Western blotting analysis (data not shown). In the case of HeLa cells, TRX is translocated from the cytoplasm to the nucleus after the stress treatment and concentrated in the nucleus for a long time [10]. In *S. cerevisiae*, however, the concentration of TRX in the nucleus was not observed under oxidative conditions.

### **Fig. 9 The intracellular localization of TRXs**

The intracellular localization of TRX was observed by immunofluorescence using the anti-yeast TRX antibody. The wild-type cells in the exponential phase were treated with or without 0.2 mM H<sub>2</sub>O<sub>2</sub> for 30 min. The increase of the level of TRX protein by H<sub>2</sub>O<sub>2</sub> (0.2 mM) was also observed by Western blotting using this antibody (data not shown).

Next, we performed two-hybrid assay experiments to examine the direct association of Yap1 with Trx1 or Trx2 (MATCHMAKER Two-Hybrid System 2, Clontech). However, we could not detect the direct association between Yap1 and Trx1 or Trx2. This might be because their association is too weak to detect by this assay system, or because TRX associates with Yap1 via other factors, such as Ref-1 in the case of AP-1 regulation [10].

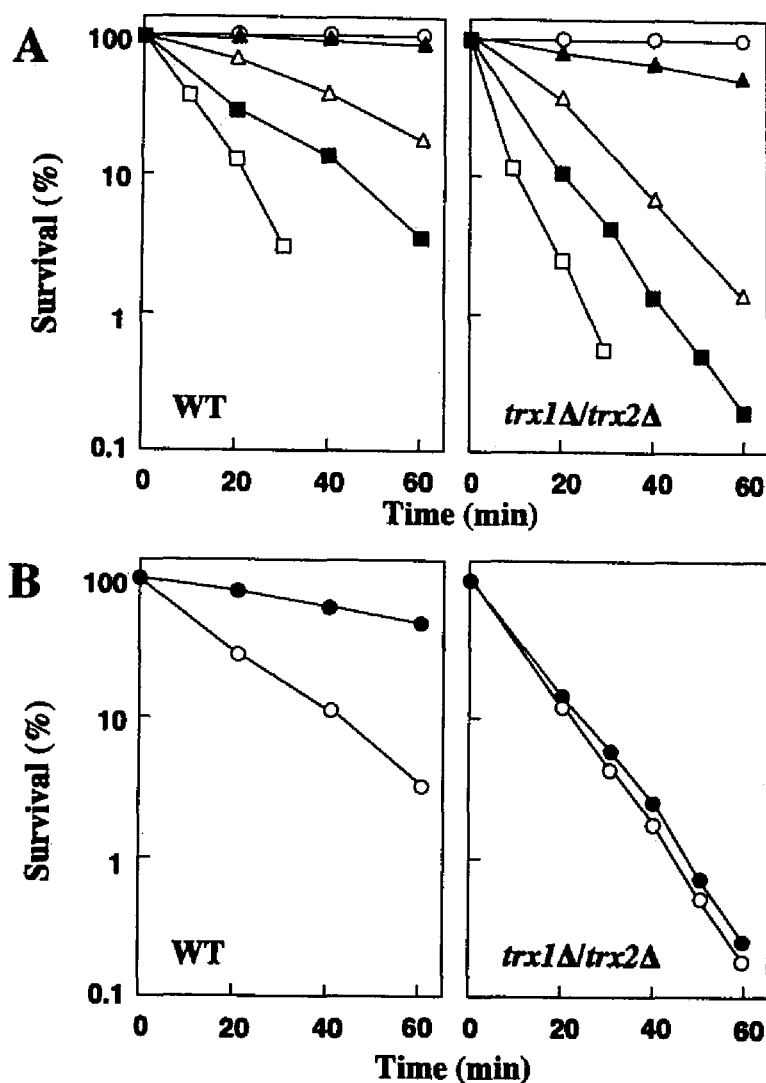
### *Synthetic Lethality of yap1 Null Allele and trx1Δ/trx2Δ*

Because the expression of the Yap1-target genes are enhanced in the *trx1Δ/trx2Δ* mutant which resulted in the increase of the activity of antioxidant enzymes, we suspected that the *trx1Δ/trx2Δ* mutant might acquire the tolerance to oxidative stress. We investigated the susceptibility to H<sub>2</sub>O<sub>2</sub> (0.2-5 mM) and the induction of adaptation to H<sub>2</sub>O<sub>2</sub> stress in *trx1Δ/trx2Δ*. The *trx1Δ/trx2Δ* mutant showed great susceptibilities to H<sub>2</sub>O<sub>2</sub> in spite of the increase of glutathione and antioxidant enzymes (Figure 10A). Furthermore, the *trx1Δ/trx2Δ* mutant was unable to induce the adaptation to H<sub>2</sub>O<sub>2</sub> despite of the constitutive activation of Yap1 (Figure 10B). The wild-type induced a large increase in tolerance to 2 mM H<sub>2</sub>O<sub>2</sub> by the pretreatment with a sublethal dose of H<sub>2</sub>O<sub>2</sub> (0.2 mM, 60 min), while the *trx1Δ/trx2Δ* mutant did not. These results indicate that the constitutive activation of Yap1 may be essential for the *trx1Δ/trx2Δ* mutant to survive under aerobic conditions rather than to acquire the resistance against oxidative stress.

In this study, we tried to construct a *yap1Δ/trx1Δ/trx2Δ* triple mutant to see the genetic interaction of these genes. We tried several times to disrupt the *TRX1* gene in YT-2a (*MATa yap1Δ::HIS3 TRX1 trx2Δ-2::LEU2*), but no viable transformant was obtained. On the contrary, it was possible to construct the *yap1Δ/grx1Δ/grx2Δ* mutant. To confirm the lethality of this triple mutant (*yap1Δ/trx1Δ/trx2Δ*), we constructed a diploid strain YT-21 (*MATa/MATα yap1Δ::HIS3/yap1Δ::HIS3 TRX1/trx1Δ::URA3 trx2Δ::LEU2/TRX2*) for tetrad analysis, by crossing YT-1α (*MATα yap1Δ::HIS3 trx1Δ::URA3 TRX2*) and YT-2a. Thirty-seven asci were subjected to tetrad analysis and all spores (37 X 4 = 148) were incubated on YPD plates at 30 °C for 2 days. Thirty-eight spores among 148 spores did not germinate. The genotype of each germinated strain (148 - 38 = 110 strains) was analyzed to guess the genotypes of the 38 spores which did not germinate. The *TRX1-TRX2* ascus type showed random assortment (parental ditype : nonparental ditype : tetratype was 6 : 7 : 24, or approximately 1 : 1 : 4 ). This result seems reasonable because the *TRX1* and *TRX2* genes locate on the different chromosomes (*TRX1* on chromosome XII and *TRX2* on chromosome VII). The genotypes of all 38 spores were inferred to be triple mutant (*yap1Δ/trx1Δ/trx2Δ*). Therefore we concluded that the combination of the *yap1Δ* mutation with the *trx1Δ/trx2Δ* mutation was lethal at least under aerobic conditions. This result also strongly indicates that the constitutive activation of



Yap1 is essential for the *trx1Δ/trx2Δ* mutant to survive under aerobic conditions.



**Fig. 10** The *trx1Δ/trx2Δ* mutant is hypersensitive to  $H_2O_2$  in spite of the constitutive activation of Yap1

(A) Cells growing exponentially in SD medium at 30°C were harvested and resuspended in 100 mM potassium phosphate buffer, pH 7.4, and treated with various concentrations of  $H_2O_2$ . Cells were withdrawn periodically, diluted with 100 mM potassium phosphate buffer and plated on YPD agar plates to monitor the cell viability.  $H_2O_2$  concentrations were: 0 (○), 0.2 (▲), 1 (△), 2 (■), 5 (□) mM, respectively. (B) Cells growing exponentially in SD medium at 30°C were harvested. ●, Cells were pretreated with 0.2 mM  $H_2O_2$  in fresh SD medium for 60 min, and then challenged to 2 mM  $H_2O_2$ . ○, Cells were challenged directly to 2 mM  $H_2O_2$ . Results represent the mean from three independent experiments.

#### 4-4. Discussion

##### *TRX in the Regulation of Yap1*

In this study, we demonstrated that Yap1 was constitutively activated and concentrated in the nucleus in *trx1Δ/trx2Δ* under non-stressed conditions. No difference was observed in the levels of Yap1 protein between the wild-type and *trx1Δ/trx2Δ*, indicating that the constitutive activation of Yap1 was due to its post-translational modification. Additionally, we also indicated the possibility that TRX negatively controls the activity of Yap1 via redox states because the constitutive activation was observed only under aerobic conditions.

It was clarified that the localization of Yap1 (and the activity of Yap1) was regulated by the nuclear export of Yap1 mediated by the interaction between Crm1 and the NES within the CRD of Yap1, and that the cysteine residue(s) within the CRD serve as redox sensors which regulate this nuclear export [29, 30]. It is conceivable that TRX affects the redox states of cysteine residue(s) within the CRD, because TRX is a reductant of disulfide bonds. TRX may reduce the cysteine residue(s) to keep the CRD in a form which is able to interact with Crm1. Therefore, Yap1 was constitutively concentrated in the nucleus in *trx1Δ/trx2Δ*, but not concentrated in the nucleus under normal conditions in the cells which contain TRX (Figure 3).

It is still controversial whether TRX directly associates with cysteine residue(s) of the CRD or not. We confirmed that TRX exists not only in the cytoplasm but also in the nucleus, especially after the treatment with a mild oxidative stress (Figure 9). Therefore, TRX would be possible to associate with Yap1 both in the cytoplasm and in the nucleus. However, we were not able to detect the direct interaction of Yap1 with Trx1 or Trx2 by the two-hybrid assay experiments. This might be because their association is too weak to detect by this assay system, or because TRX cannot associate directly with Yap1. If TRX has no direct association with Yap1, TRX may affect the redox states of the CRD via other factors, such as Ref-1 in the case of AP-1 regulation [10]. Yan *et al.* reported that a single cysteine residue within the CRD is necessary and sufficient for the response to oxidative stress, and speculated that the cysteine residue(s) within the CRD form linkages with a third protein, as yet unidentified, which serves to mask the NES [30]. TRX may regulate the formation of these linkages. Interestingly, the *TRX2* gene is one of the target genes for Yap1. Therefore, Yap1 may be

autoregulated; active form of Yap1 enhances transcription of the *TRX2* gene, and Trx2 may promote the interaction between Crm1 and Yap1 by unmasking the NES to export Yap1, as one of the down regulations. This idea may be supported by the result that TRX exists in the nucleus under the mild stress conditions (Fig. 9).

Another possible explanation is that TRX may take part in the regulation of Yap1 via the redox states of the whole intracellular environments. TRX not only modifies proteins such as transcription factors, but also maintains intracellular environments in reduced states [11, 12]. The deficiency of the thioredoxin system causes the oxidation of the cytoplasm in *E. coli* (12). The TRX-deficiency in yeast may change the intracellular redox states, and consequently this change would cause the redox modification of cysteine residues of the CRD and the activation of Yap1. Indeed, we showed that the level of intracellular oxidation in *trx1Δ/trx2Δ* was higher than that of the wild-type (Figure 4B), and it has been reported that the *trx1Δ/trx2Δ* mutant accumulates high levels of oxidized glutathione [47]. These results support the possibility of the change in intracellular redox states caused by TRX-deficiency in *S. cerevisiae*.

Taken together with the results in this study, we present a tentative model for the roles of TRX in the regulation of Yap1 (Figure 11). Under normal conditions, TRX reduces intracellular environments and may maintain the CRD in a reduced form which can interact with Crm1. After the stimulation with a mild oxidative stress, the synthesis of TRX is induced by the activated Yap1, and then TRX may function as a deactivator for Yap1 besides as an antioxidant enzyme (Figure 12). TRX may unmask the NES and facilitate the interaction between the CRD and Crm1. TRX in the regulation of Yap1 in yeast may play a similar role of GRX in the deactivation of OxyR in *E. coli* [3]. The details of the interaction between Yap1 and TRX are currently under investigation.

### *The Difference in Functions between TRX and GRX*

In addition to TRX, GRX is also a reductant of disulfide bonds, and known as a redox regulator of OxyR in *E. coli* [3]. It has been reported that both TRX and GRX can partially substitute for each other *in vivo*, and contribute to maintaining the cytoplasm in a reduced state in *E. coli* [12, 49]. Both of these thiol-disulfide oxidoreductases are required for protection

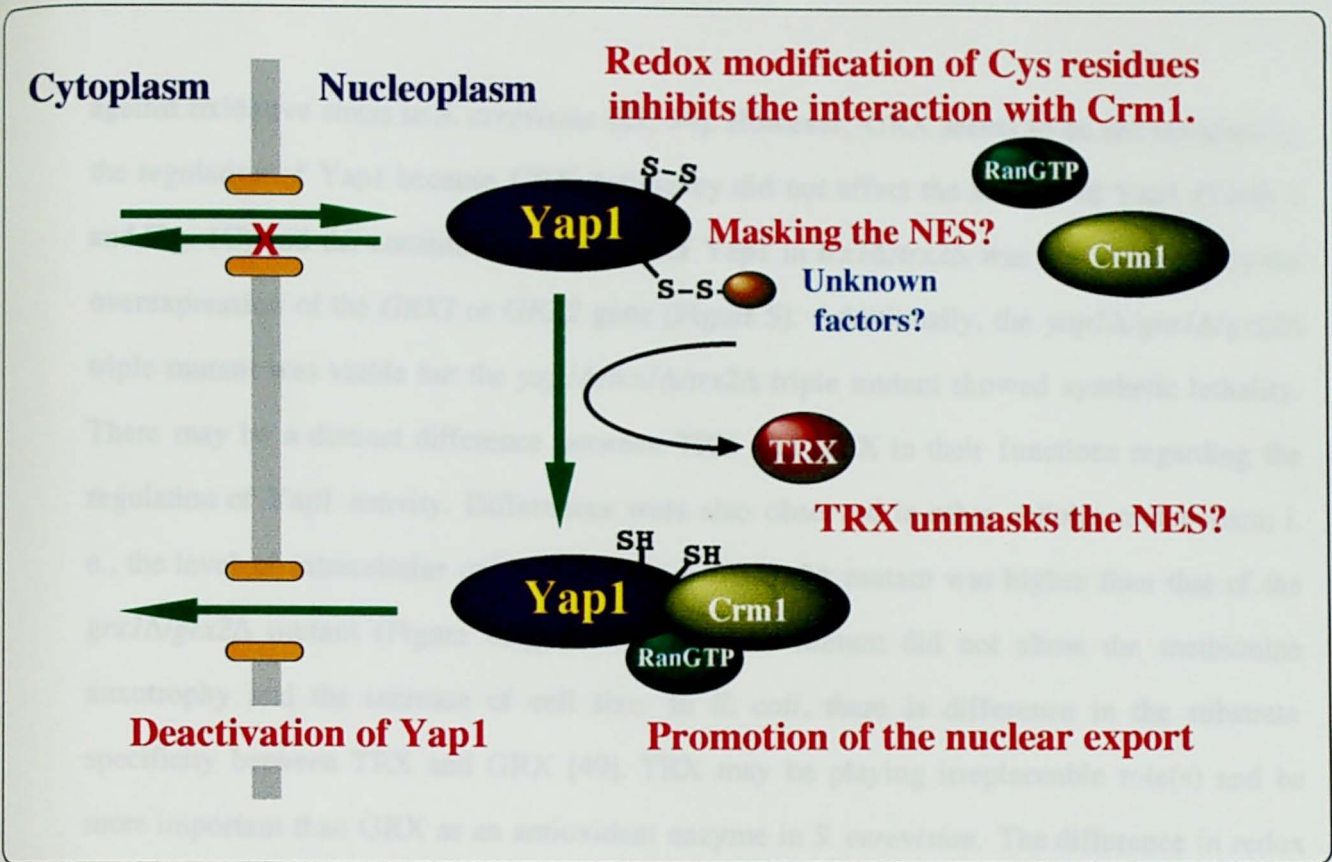


Fig. 11 A tentative model: regulation of Yap1 by TRX

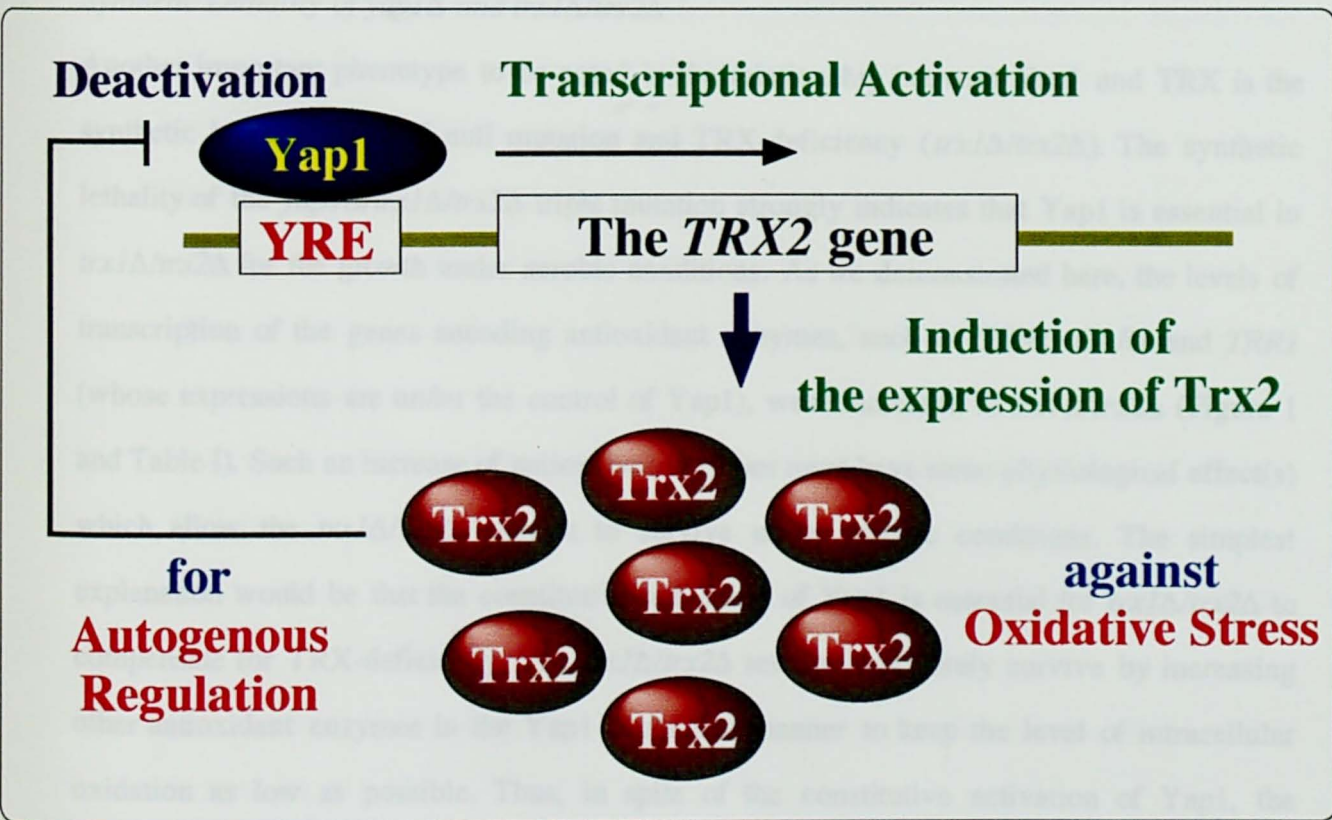


Fig. 12 Deactivation of Yap1 by Thioredoxin 2

against oxidative stress in *S. cerevisiae* [21, 34]. However, GRX seems to be not involved in the regulation of Yap1 because GRX-deficiency did not affect the activity of Yap1 (Table 1 and Fig. 1C) and the constitutive activation of Yap1 in *trx1Δ/trx2Δ* was not repressed by the overexpression of the *GRX1* or *GRX2* gene (Figure 5). Additionally, the *yap1Δ/grx1Δ/grx2Δ* triple mutant was viable but the *yap1Δ/trx1Δ/trx2Δ* triple mutant showed synthetic lethality. There may be a distinct difference between TRX and GRX in their functions regarding the regulation of Yap1 activity. Differences were also observed in other cellular metabolism; i. e., the level of intracellular oxidation of the *trx1Δ/trx2Δ* mutant was higher than that of the *grx1Δ/grx2Δ* mutant (Figure 4B), and *grxΔ/grx2Δ* mutant did not show the methionine auxotrophy and the increase of cell size. In *E. coli*, there is difference in the substrate specificity between TRX and GRX [49]. TRX may be playing irreplaceable role(s) and be more important than GRX as an antioxidant enzyme in *S. cerevisiae*. The difference in redox potential may be one of causes of the difference in functions between TRX and GRX [51], but it is still obscure what the cause of the difference in functions is.

#### *Synthetic Lethality of yap1Δ and trx1Δ/trx2Δ*

Another important phenotype to be noted in the relationship between Yap1 and TRX is the synthetic lethality of *yap1* null mutation and TRX-deficiency (*trx1Δ/trx2Δ*). The synthetic lethality of the *yap1Δ/trx1Δ/trx2Δ* triple mutation strongly indicates that Yap1 is essential in *trx1Δ/trx2Δ* for the growth under aerobic conditions. As we demonstrated here, the levels of transcription of the genes encoding antioxidant enzymes, such as *GSH1*, *GLR1* and *TRR1* (whose expressions are under the control of Yap1), were increased in *trx1Δ/trx2Δ* (Figure 1 and Table I). Such an increase of antioxidant enzymes must have some physiological effect(s) which allow the *trx1Δ/trx2Δ* mutant to survive under aerobic conditions. The simplest explanation would be that the constitutive activation of Yap1 is essential for *trx1Δ/trx2Δ* to compensate for TRX-deficiency. The *trx1Δ/trx2Δ* mutant may barely survive by increasing other antioxidant enzymes in the Yap1-dependent manner to keep the level of intracellular oxidation as low as possible. Thus, in spite of the constitutive activation of Yap1, the *trx1Δ/trx2Δ* mutant was hypersensitive to H<sub>2</sub>O<sub>2</sub> (Figure 10) and its intracellular oxidation level was still higher than that of the wild-type (Figure 4B). The synthetic lethality of

*yap1Δ/trx1Δ/trx2Δ* presumably reflects the serious intracellular redox states. The intracellular oxidation level of *grx1Δ/grx2Δ* was almost the same with that of the wild-type (Figure 4B), and the *yap1Δ/grx1Δ/grx2Δ* triple mutant was viable. The disruption of the *GLR1* gene was also lethal in the *trx1Δ/trx2Δ* background under aerobic conditions [47]. These observations may support our idea.

#### 4-5. Acknowledgments

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1. The first part of the paper discusses the importance of the study of the history of the English language in the context of the development of the English language as a world language. It also discusses the importance of the study of the history of the English language in the context of the development of the English language as a world language.

## General Conclusion

### Chapter 1

Controversy about the significance of catalase in detoxification of  $H_2O_2$  in human erythrocytes continues today. It has been suggested that catalase has no role in the clearance of  $H_2O_2$  in erythrocytes. In this chapter, the author investigated the role of catalase in the defense mechanism against oxidative stress using *Saccharomyces cerevisiae*. *S. cerevisiae* has two catalases, catalase A and catalase T. The author constructed a double mutant (catalase-deficient mutant) unable to produce either catalase A and catalase T, and compared it with wild-type and single mutant cells. The catalase-deficient mutant cells showed the similar growth rate with wild-type cells under non-stressed conditions, and showed the similar susceptibility against  $H_2O_2$  stress in exponentially growth phase. The catalase-deficient mutant cells of stationary phase were, however, much more sensitive against  $H_2O_2$  stress than wild-type cells and single mutant cells. Moreover, the ability of catalase-deficient and single-mutant cells to show the adaptation to 2 mM  $H_2O_2$  was distinctly inferior to that of wild-type cells. These results suggest that catalase is not essential for yeast cells under normal conditions, but plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of yeast cells.

### Chapter 2

Role of intracellular glutathione in the response of *Saccharomyces cerevisiae* to  $H_2O_2$  was investigated. Depletion of cellular glutathione or inhibition of  $\gamma$ -glutamylcysteine synthetase (GSH-I) enhanced the sensitivity and suppressed the adaptation to  $H_2O_2$ . A mutant deficient in GSH-I also showed the hypersensitivity and could not adapt to  $H_2O_2$ . Incubation of the cell with amino acids constituting glutathione (L-Glu, L-Cys, Gly) increased the intracellular glutathione content, and subsequently the cell acquired the resistance against  $H_2O_2$ . These results strongly suggest that intracellular glutathione plays an important role in the adaptive response in *S. cerevisiae* to oxidative damage.

### Chapter 3

Glucose-6-phosphate dehydrogenase (G6PDH)-deficient cells of *Saccharomyces cerevisiae* showed increased susceptibility and were unable to induce adaptation to oxidative stress. Historically, mainly in human erythrocytes, it has been suggested and accepted that decrease of the cellular reduced form glutathione (GSH), due to loss of the NADPH-dependent activity of glutathione reductase (GR), is responsible for the increased sensitivity to oxidative stress in G6PDH-deficient cells. In this chapter, the author investigated whether the increased susceptibility and the inability to induce adaptation to  $H_2O_2$  stress of G6PDH-deficient yeast is caused by incompleteness of glutathione recycling. The author constructed G6PDH- and GR-deficient mutants and analyzed their adaptive response to  $H_2O_2$  stress. Although G6PDH-deficient cells contained comparable amounts of GSH and GR activity to wild-type cells, the oxidized form glutathione (GSSG) was not reduced efficiently, and intracellular GSSG levels and the ratio of GSSG to total glutathione (GSSG/tGSH) were higher in G6PDH-deficient cells than in the wild-type. On the other hand, GR-deficient cells showed an identical susceptibility with that of wild-type cells and induced adaptation to  $H_2O_2$  stress, even though the GSSG/tGSH ratio in GR-deficient cells was higher than in G6PDH-deficient cells. These results indicate that incompleteness of glutathione recycling alone is not sufficient to account for increased sensitivity and inability to induce adaptation to  $H_2O_2$  stress of G6PDH-deficient yeast cells. In *S. cerevisiae*, G6PDH seems to be playing other important roles in the adaptive response against  $H_2O_2$  stress, besides supplying NADPH to GR reaction.

### Chapter 4

Yap1 is a transcription factor which responds to oxidative stress in *Saccharomyces cerevisiae*. The activity of Yap1 is regulated at the level of its intracellular localization, and a cysteine rich domain at the C-terminus of Yap1 is involved in this regulation. The author investigated the effects of redox-regulatory proteins, thioredoxin and glutaredoxin, on the regulation of Yap1, using the deficient mutants of these thiol-disulfide oxidoreductases. In the thioredoxin-deficient mutant (*trx1Δ/trx2Δ*), Yap1 was constitutively concentrated in the nucleus and the level of expression of the Yap1-target genes was high under normal conditions, while this was not the case for the glutaredoxin-deficient mutant (*grx1Δ/grx2Δ*). No distinct difference

was observed in the levels of Yap1 protein between the wild-type and *trx1Δ/trx2Δ*. The constitutive activation of Yap1 in *trxΔ/trx2Δ* was observed under aerobic conditions but not under anaerobic conditions. These findings suggest that thioredoxin has negative effects on this regulation via the redox states. The three cysteine residues near the NES in the CRD (Cys<sup>598</sup>, Cys<sup>629</sup>, and Cys<sup>620</sup>) were playing important roles in the regulation of the nuclear export of Yap1, and thioredoxin seems to regulate the redox states of these cysteine residues. Taken together with the results in this study, the author presented a tentative model for the roles of TRX in the regulation of Yap1. The author also showed the synthetic lethality between *yap1Δ* and *trx1Δ/trx2Δ* mutation, but the *yap1Δ/grx1Δ/grx2Δ* triple mutant was viable, suggesting a difference of the functions between thioredoxin and glutaredoxin and a genetic interaction between Yap1 and thioredoxin *in vivo*.

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- 2 S. Izawa, Y. Inoue, and A. Kimura (1996) Importance of catalase in the adaptive response to hydrogen peroxide: analysis of catalase-deficient *Saccharomyces cerevisiae* *Biochem. J.* **320** (1), 61-67
- 3 S. Izawa, K. Maeda, T. Miki, J. Mano, Y. Inoue, and A. Kimura (1998) Importance of glucose-6-phosphate dehydrogenase in the adaptive response to hydrogen peroxide in *Saccharomyces cerevisiae* *Biochem. J.* **330** (2), 811-817
- 4 S. Izawa, K. Maeda, K. Sugiyama, J. Mano, Y. Inoue, and A. Kimura (1999) Thioredoxin-deficiency causes the constitutive activation of Yap1, an AP-1 like transcription factor in *Saccharomyces cerevisiae* (Submitted for publication)